EVALUATION OF BROMINE FOR DISINFECTION OF DRINKING WATER

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ABSTRACT

JOHN JIHOON KIM: Evaluation of Bromine for Disinfection of Drinking Water
(Under the direction of Mark D. Sobsey)

Conventional water treatment practices utilizing chemical disinfection, primarily chlorination, are considered generally effective in producing microbiologically safe drinking water. However, some pathogens, such as oocysts of the protozoan *Cryptosporidium parvum*, are very resistant to chlorine, which has led to consideration of alternative disinfectants for its control.

The objective of this study was to compare the efficacies of free chlorine and bromine in the disinfection of MS-2 coliphages, *Bacillus atrophaeus* spores, and *Cryptosporidium parvum* oocysts; and to investigate suitable models for the kinetics of inactivation of these microorganisms. The halogens were dosed separately at 5 mg/L to batch reactors seeded with the microorganisms in phosphate buffered (pH 7.5, a normally-occurring pH) halogen-demand free water. Experimental waters were held at 25°C and sampled periodically for up to 1000 minutes. Disinfection efficacy was evaluated by the reductions in culturable or infectious test organisms as determined by their respective microbial assays.

Chlorine was a more efficacious disinfectant than bromine against MS-2 and *Bacillus* spores. MS-2 inactivation was rapid for both halogens and exhibited first-order inactivation kinetics; CT values to achieve 4 $\log_{10}$ reduction were $<$3.8 min*mg/L and 19

$\pm$ 2.5 min*mg/L for chlorine and bromine, respectively. *Bacillus* spore inactivation exhibited shouldering characteristics and was well-characterized by the Hom model ($n = 1, m > 1$). At a 5 mg/L dose, chlorine and bromine achieved 4 $\log_{10}$ reductions in spores at around 32 and 440 minutes, respectively.

Chlorine treatment at 5 mg/L enhanced *C. parvum* oocyst infectivity. A two-fold increase in infectivity occurred within 5 minutes, but the rate of "activation" quickly declined thereafter. This trend was best described by the Hom model ($n = 1, m < 1$). Bromine treatment at 5 mg/L decreased oocyst infectivity. A 50% reduction was achieved quickly within 4 minutes, followed by a very slow increase in infectivity. The largest observed reduction was 84%. The data were best modeled by the One Hit—Two Population model, suggesting the presence of two oocyst populations with different levels of susceptibility to bromine.

The research demonstrates that chlorine and bromine can each achieve greater than a 5-$\log_{10}$ reduction of MS-2 and *Bacillus* spores at a dose of 5 mg/L, although chlorine can achieve the same reductions in a shorter time period than bromine. Also, at 5 mg/L, neither chlorine nor bromine is an adequate disinfectant against *C. parvum* oocysts, although bromine does achieve limited disinfection.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

1 INTRODUCTION

Previous research on microbial inactivation by bromine has indicated that bromine is a strong disinfectant and is suitable for disinfecting waters of various types. The majority of these studies were conducted in the 1970s as a range of alternatives to chlorine were being sought. Since then, there have been few investigations of bromine as a water disinfectant because of concerns about brominated disinfection byproducts. However, interest in this topic has recently renewed. The research presented in this report was undertaken to evaluate the performance of bromine as a drinking water disinfectant by determining its effectiveness to reduce concentrations of infectious *Cryptosporidium parvum* oocysts, coliphage MS-2, and spores of *Bacillus atrophaeus* in buffered, halogen-demand free water.

1.1 OBJECTIVES

The objectives of the research presented in this report are twofold: (1) to compare the efficacies of free chlorine and bromine in the disinfection of MS-2 coliphages, *Bacillus atrophaeus* spores, and *Cryptosporidium parvum* oocysts; and (2) to investigate suitable models for the kinetics of inactivation of these microorganisms.

1.2 APPROACH

Lab-scale batch disinfection experiments were performed to achieve the first objective. The halogens were dosed separately to batch reactors seeded with the microorganisms in phosphate buffered (pH 7.5) halogen-demand free water. Samples

were taken from the reaction vessels at specified intervals for up to 1000 minutes. Residual halogen concentrations were also measured. Disinfection efficacy was evaluated by the reductions in culturable or infectious test organisms as determined by their respective microbial assays.

To address the second objective, inactivation kinetics curves were developed from the microbial survival data as a function of contact time. The data were fitted for different disinfection kinetic models, and the most predictive and plausible models were selected. The models were then used to estimate the combination of halogen concentration and contact time required to achieve certain levels of microbial inactivation, providing more reliable estimates relevant to the first objective.

The remainder of this report is structured as follows. Chapter 2 begins with a brief overview of centralized water treatment practices; then addresses lab-based evidence of the resistance of *C. parvum* oocysts to chlorine disinfection; then provides a detailed synthesis of the current understanding of bromine disinfection for different water uses; and finally ends with relevant approaches to disinfection kinetics modeling. Chapter 3 presents the details of the materials and methods used in lab experiments and the subsequent data analysis. Chapter 4 presents the experimental findings and compares kinetic models. Chapter 5 is a discussion of the results, providing implications of those results and recommendations for future research. Chapter 6 concludes the report with the key findings.

2 BACKGROUND

Drinking water treatment plants are essential to the protection of public health because they serve as the primary barrier against waterborne pathogens. In the United States, water treatment plants typically involve coagulation, flocculation, filtration, and chemical disinfection. The continuous chlorination of drinking water was first introduced in the U.S. in 1908 in Jersey City, New Jersey (Leal, 1909). Since then, the importance of chemical disinfection has become widely accepted. Commonly used disinfectants are free chlorine, monochloramine, ozone, and chlorine dioxide. UV treatment is utilized as well, but because it provides no protective disinfectant residual in the distribution system, a chemical disinfectant is typically added before the water enters the distribution system.

2.1 CHLORINE DISINFECTION OF C. PARVUM OOCYSTS

It is well-known that *C. parvum* oocysts are highly resistant to chlorine and chloramine disinfection. The American public became hyperaware of this fact after the Milwaukee Cryptosporidiosis outbreak of 1993. At the time, the Milwaukee Water Works (MWW) system provided drinking water to consumers through two drinking water treatment plants, Linwood (north plant) and Howard Avenue (south plant). Raw water was pumped from Lake Michigan into both plants. The treatment processes were identical in both: chemical coagulation, followed by rapid sand filtration, and chloramine disinfection. Retrospective investigations of the outbreak indicated deficiencies in the coagulation and filtration processes of the southern plant at the time of the outbreak. The

final barrier, chloramine disinfection, was unfortunately completely ineffective against *Cryptosporidium*. As a result, an estimated 403,000 people suffered from watery diarrhea attributable to the outbreak (MacKenzie, et al., 1994), and 54 cryptosporidiosis-associated deaths were identified during the two-year post-outbreak period (Hoxie et al., 1997), making this the largest recorded waterborne outbreak in United States history.

While the outbreak served to raise awareness of the resistance of *Cryptosporidium* to chlorine treatment, laboratory researchers had been investigating the topic for several years prior. Based on mouse infectivity trials, Korich et al. (1990) found that at least 99% inactivation (2 $\log_{10}$ reduction) of infectious oocysts was achieved after 90 minutes of exposure to 80 mg/L of chlorine, corresponding to a CT value of 7,200 (min*mg/L). Fayer (1995) found that developmental stages of *C. parvum* were present in all mice orally dosed with *Cryptosporidium*, even after the oocysts were exposed to 5.25, 2.63, and 1.31% aqueous sodium hypochlorite for two hours. Venczel et al. (1997) found that after 24 hours of exposure to 5 mg/L free chlorine (0.01 M phosphate buffer, pH 7, 25°C), oocyst infectivity (mouse trials) was essentially unaffected.

Some of these findings appear to conflict, with some research groups concluding that free chlorine was essentially ineffective against *C. parvum* oocysts, and others finding that free chlorine was somewhat effective, albeit not enough to consider chlorine a sufficient barrier against *C. parvum* oocysts. There are two possible reasons for the disagreements in the literature. Firstly, the methods used to assess the viability or infectivity of the oocysts were not identical. In just the studies mentioned in this review, several assays were used: mouse infectivity, cell culture infectivity, cell culture PCR, in vitro excystation, and vital dye stains. These methods do not necessarily measure the

same things, and researchers are always at the mercy of the limitations of the models that are available.

2.2 LITERATURE REVIEW OF WATER TREATMENT APPLICATIONS OF BROMINE

2.2.1 Background

There was considerable new research on bromine for water and wastewater disinfection in the 1970s as a range of alternatives to chlorine was being sought. However, the momentum on bromine disinfection research for water and wastewater soon dissipated after brominated disinfection byproducts were reported to be detrimental to human health (White, 1998). Consequently, most of the known research and development of bromine for water and wastewater treatment occurred almost half a century ago. Since then, there have been few investigations of bromine as a water disinfectant, although interest in this topic has recently renewed.

There has only been one documented case of the use of free bromine in a municipal drinking water supply, which was in Irvington, California circa 1938 (White, 1998). However, its use was discontinued because of its inability to maintain a residual due to bromine's high reactivity with organic constituents in the distribution system. Since then, bromine has been used regularly in swimming pools (Brown & McLean, 1966; Kelsall & Sim, 2001) and cooling towers (Kim et al., 2002). In recent years, bromine was found to be the second most common biocide used as a disinfectant in a survey of over 6,000 cooling towers located throughout the United States (Miller et al., 2011). In 2010, a new gravity-driven household point-of-use drinking water system utilized N-halamine bromine or chlorine as its disinfection media. Coulliette et al. (2010)

indicated that the bromine canisters consistently outperformed the chlorine canisters in reducing microcystin toxins as well as surpassing the virus reduction goal of 99.99% set forth by the U.S. Environmental Protection Agency. Therefore, it seems there is renewed interest in bromine as more than just an industrial water disinfectant, including possible use as a drinking water disinfectant.

This literature review aims to summarize the accumulated knowledge of the essential chemistry and disinfection capabilities of bromine and its various chemical species as a microbicide for water treatment applications.

2.2.2 Bromine Chemistry

As members of the same halogen family, bromine and chlorine undergo somewhat similar chemical interactions in water. For instance, the hydrolysis of aqueous bromine is given as follows:

$$Br_2(aq) + H_2O \rightleftarrows HOBr + Br^- + H^+$$

with an equilibrium constant estimated to be K = (3.5 ± 0.1) x 10^{-9} at 25° C (Beckwith, Wang, & Margerum, 1996) or K = 5.8 x 10^{-9} (White, 1998). Thus, when molecular bromine is added to water, it is quickly converted to hypobromous acid (HOBr). This is similar to how hypochlorous acid (HOCl) is formed quickly when molecular chorine is added to water. Additionally, both HOBr and HOCl will dissociate further as follows:

$$HA \rightleftarrows H^+ + A^-$$

where *HA* is the hypohalous acid and A^- is the hypohalite ion. The pK_a value (which is the pH at which the acid and the conjugate base are at equimolar concentrations) for HOBr is 8.7, and that for HOCl is 7.5. It is well known that hypochlorous acid is a stronger microbicide than its conjugate base; according to one estimate, HOCl is about 80

to 200 times stronger as an oxidant than OCl^- (Droste, 1997). This is crucial for water and wastewater treatment plant operations, as the pH of the water must be maintained at a level optimal for disinfection (typically, below pH 7.5). In this regard, free bromine has a wider "effective" pH window than free chlorine in natural waters, prior to any artificial pH adjustments.

A special characteristic of bromine chemistry is that, in the presence of free chlorine, ambient bromide ions are oxidized to HOBr very rapidly:

$$HOCl + Br^- \rightarrow HOBr + Cl^-$$

where $k_1 = 1550\ M^{-1}s^{-1}$ (Kumar & Margerum, 1987). This reaction was also previously noted in Farkas et al. (1949). In water treatment plants that utilize chlorine disinfection and have any level of bromide content in their receiving waters, free bromine is produced "in situ" (i.e., during the disinfection process). Corresponding to the extent of bromide ion content, free bromine becomes the disinfectant rather than free chlorine. This is a phenomenon well-documented in literature of the transformation and degradation of pharmaceutical and personal care products (Lee & Von Gunten, 2009; Acero et al., 2011). Thus, bromine studies should be viewed as a necessary corollary of chlorine disinfection studies in order to optimize operations in treatment plants that have appreciable bromide content in their influent water.

Bromine chemistry is further complicated by the presence of ammonia in the water. Free bromine combines quickly with ammonia to form bromamines, mostly dibromamine ($NHBr_2$) and tribromamine (NBr_3). However, dibromamine decomposes rapidly, so much of the initially added bromine (and its corresponding disinfecting power) is lost almost instantaneously (Johnson & Overby, 1970).

The interhalogen, bromine chloride (BrCl), has also been a subject of disinfection studies, mainly for wastewater disinfection. BrCl is prepared by adding equivalent moles of bromine and chlorine:

$$Br_2 + Cl_2 \rightleftarrows 2\ BrCl$$

The disinfecting reactivity of BrCl comes from the larger halogen bromine. It appears that BrCl hydrolyzes only to HOBr (Mills, 1975):

$$BrCl + H_2O \rightarrow HOBr + HCl$$

with a hydrolysis constant of 2.94 x 10^{-5} at 0° C, which is several orders of magnitude greater than that of bromine as reported above. Consequently, it can be inferred that when BrCl is utilized in disinfection, the reactive species is actually the hydrolysis product HOBr.

2.2.3 Lab Studies on Bromine Disinfection

A number of lab-scale bromine disinfection studies were performed in the 1970s, with a few experiments performed as early as the 1930s. Some studies were done using oxidant demand-free water, and others were done using a wastewater matrix. Particular attention was given to the different bromine species that arose from the different test waters. Findings from these studies are separated by halogen species—free bromine, bromamines, and bromine chloride—and are compiled in the tables in the following subsections.

2.2.3.1 Free Bromine

There is much evidence that free bromine is an effective disinfectant against a variety of viruses, bacteria and protozoa, as shown in Table 1 through Table 3.

Table 1: Inactivation of Microorganisms by Free Bromine

Microorganism	Water	Halogen Species	Dose	Residual (mg/L)	Temp (°C)	pH	Cont. Time	Kill	Reference
Proteus vulgaris	N.D.[1]	HOBr	40-60 mg/L	N.D.	N.D.	3.5-4.0	30 s	C.K.[2]	Tanner &
			170 mg/L			6.8-7.2	30 s	C.K.	Pitner, 1939
Bacillus		HOBr	28-35 mg/L	N.D.	N.D.	3.5-4.0	30 s	C.K.	
megatherium			110 mg/L			6.8-7.2	30 s	C.K.	
Bacillus spp.[3]		HOBr	160-220 mg/L	N.D.	N.D.	3.5-4.0	30 s	C.K.	
			> 450 mg/L			6.8-7.2	30 s	C.K.	
Aspergillus spp.[4]		HOBr	25-28 mg/L	N.D.	R.T.[5]	N.D.	15-30 s	C.K.	
Oöspora lactis		HOBr	8 mg/L	N.D.	R.T.	N.D.	15-30 s	C.K.	
Mucor sp.		HOBr	30 mg/L	N.D.	R.T.	N.D.	15-30 s	C.K.	
Penicillium sp.		HOBr	1-5 mg/L	N.D.	R.T.	N.D.	15-30 s	C.K.	
Yeasts[6]		HOBr	0.25-0.5 mg/L	N.D.	R.T.	N.D.	15-30 s	C.K.	
Saccharomyces sp.		HOBr	3.0 mg/L	N.D.	R.T.	N.D.	15-30 s	C.K.	
Staphylococcus spp.[7]		HOBr	0.10-0.25 mg/L	N.D.	R.T.	N.D.	15-30 s	C.K.	
E. coli[8]		HOBr	0.15 mg/L	N.D.	R.T.	N.D.	15-30 s	C.K.	
Eberthella spp.[9]		HOBr	0.03-0.06 mg/L	N.D.	R.T.	N.D.	15-30 s	C.K.	

[1] N.D. = no data
[2] C.K. = "complete kill"
[3] Species tested: *Bacillus mesentericus, subtilis* (565*), subtilis* (566)
[4] Species tested: *Aspergillus niger*, sp.
[5] R.T. = room temperature
[6] Genera and species tested: *Cryptococcus*, *Mycoderma*, *Monila albicans*
[7] Species tested: *Staphylococcus aureus* (92), *albus* (76), sp. (80), *aureus* (77), *aureus* (79), *aureus* (89)
[8] Strains tested: *Escherichia coli* (252), (251)
[9] Species tested: *Eberthella typhosa* (377), (378)

Table 2: Inactivation of Microorganisms by Free Bromine (continued)

Microbe	Water	Halogen Species	Dose	Residual (mg/L[1])	Temp (°C)	pH	Cont. Time	Log_{10} Red.	Reference
Total bacterial count	SPW[2]	HOBr	No Data	0.2	N.D.[3]	7.7-7.8	N/A[4]	340[5]	Goodenough, 1964
						7.5-7.6		>400	
						7.2-7.3		54	
						7.0-7.1		40	
	SPW	HOBr	No Data	0.4	N.D.	7.7-7.8	N/A	140	
						7.5-7.6		250	
						7.2-7.3		32	
						7.0-7.1		20	
	SPW	HOBr	No Data	0.6	N.D.	7.7-7.8	N/A	13	
						7.5-7.6		38	
						7.2-7.3		19	
						7.0-7.1		10	
	SPW	HOBr	No Data	0.8	N.D.	7.7-7.8	N/A	10	
						7.5-7.6		10	
						7.2-7.3		8	
						7.0-7.1		2	
f2 phage	PB[6]	HOBr	4 mg/L	N.D.	0	7.5	~1 m	~ 2.3	Lindley, 1966
							~3 m	~ 2.6	
							5 m	~ 3.5	
							20 m	~ 4.5	
							30 m	~ 4.5	
E. coli	PB	HOBr	4 mg/L	N.D.	0	7.5	5 m	~ 2.7	
							10 m	~ 3.7	
							20 m	~ 4.5	
							30 m	~ 4.5	
f2 phage	PB	HOBr	4 mg/L	3.4	0	7.5	10 m	~ 5.0	
			6 mg/L	2.9				~ 4.0	
			8 mg/L	1.35				~ 3.3	
E. coli	PB	HOBr	4 mg/L	N.D.	0	7.5	10 m	~ 4.0	
E. coli	N.D.	HOBr/ Br_2	4 mg/L	N.D.	0	6.0	10 m	4.7	Krusé et al., 1970
		HOBr	4 mg/L			7.0		5	
		HOBr/OBr^-	4 mg/L			8.0		3.2	
f2 phage	N.D.	HOBr/ Br_2	4 mg/L	N.D.	0	6.0	10 m	6.5	
		HOBr	4 mg/L			7.0		3.7	
		HOBr/OBr^-	4 mg/L			8.0		2.5	

[1] All mg/L measurements are concentrations as Br_2
[2] SPW = swimming pool water
[3] N.D. = no data
[4] N/A = not applicable, equilibrium conditions may be assumed (i.e., sufficient contact time)
[5] Total bacterial plate count per mL of SPW (not log_{10} reductions), for all Goodenough (1964)
[6] PB = phosphate buffered water

Table 3: Inactivation of Microorganisms by Free Bromine (continued)

Microbe	Water	Halogen Species	Dose	Residual (mg/L[1])	Temp (°C)	pH	Cont. Time	Log_{10} Red.	Reference
f2 phage	PBDF[2]	HOBr	15 mg/L	No Data	0	7.5	~5 m	~ 3.4	Olivieri et al., 1975
Reovirus III	PBDF	HOBr	2.5 µM	No Data	~ 2	7.0	3 m	~ 2.3	Sharp et al., 1975
			4.7 µM				3 m	~ 3.1	
			5.4 µM				1 m	~ 3.6	
			7 µM				30 s	~ 3.4	
Total coliform	SWWE[3]	(HOCl)+HOBr	1.5 mg/L	0.18	N.D.[4]	7.5	20 m	1.7	Sollo et al., 1975
		HOBr	1.5 mg/L	0.09	N.D.	9.0	20 m	0.7	
E. histolytica Cyst	PBDF	HOBr	No Data	1.5	30	4	10 m	3	Stringer et al., 1975
				4	N.D.	10	10 m	3	
Polio I	PBDF	HOBr	0.6 µM	No Data	2	7	16 s	~ 1	Floyd et al., 1976
			2.2 µM				16 s	~ 1.8	
			22 µM				16 s	~ 3.8	
Polio I	PBDF	HOBr	1.9 µM	No Data	10	7	16 s	~ 1	
			5.9 µM				16 s	~ 3	
			10 µM				12 s	~ 3.4	
Polio I	PBDF	HOBr	1.9 µM	No Data	20	7	8 s	~ 2.3	
			5.5 µM				6 s	~ 3	
			9.5 µM				6 s	~ 3.2	
Polio I	PBDF	OBr^-	4.3 µM	No Data	2	11	4 s	2.1	Floyd et al., 1978
			5.5 µM				4 s	~ 3.5	
			9.6 µM				4 s	3.85	
Polio I	PBDF	OBr^-	2 µM	No Data	4	10	4 s	~ 1.9	
			6 µM				4 s	~ 2.2	
			12 µM				4 s	~ 2.3	
Polio I	PBDF	Br_2	4.7 µM	No Data	4	5	4 s	~ 3.2	
	+ 0.3 M		12.9 µM				2 s	~ 3.4	
	NaCl		21.6 µM				2 s	~ 4.4	
Polio I	PBDF	Br_2	12.9 µM	No Data	4	5	4 s	~ 3.7	
	+ 0.3 M		19.7 µM				3 s	~ 3.5	
	NaCl		21.6 µM				1.5 s	~ 3.8	
P. aeruginosa[5]	PBDF	HOBr[6]	0.8 mg/L	No Data	38	7.5	10 m	> 2	Clark & Smith, 1992
			1.5 mg/L				0.5 m	> 2	
P. aeruginosa[7]	PBDF	HOBr	0.2 mg/L	No Data	38	7.5	0.5 m	> 2	
P. aeruginosa[5]	PBDF	DiHalo	0.8 mg/L	No Data	38	7.5	10 m	> 2	
			1.5 mg/L				1 m	> 2	
			2.2 mg/L				0.5 m	> 2	
P. aeruginosa[7]	PBDF	DiHalo	0.2 mg/L	No Data	38	7.5	0.5 m	> 2	

[1] All mg/L measurements are concentrations as Br_2
[2] PBDF = phosphate buffered demand free water
[3] SWWE = secondary treated wastewater effluent
[4] N.D. = no data
[5] Natural (environmental) strain
[6] Not explicit; interpreted as HOBr
[7] Lab strain

Tanner & Pitner (1939) performed some early work on the germicidal action of bromine. They tested free bromine against a variety of organisms (aerobic spore-forming bacteria, mold spores, yeasts, and non-spore-forming bacteria). The amount of free bromine required to "kill" (i.e., reduce beyond detection limits) each species in a given time interval was reported (initial titer was not reported). For each aerobic spore-forming bacterium tested, typically three to four times more bromine was required to kill the bacteria in 30 seconds at neutral pH (6.8-7.2) than at the lower pH (3.5-4.0). At the lower pH, 40-60 ppm of bromine was required for *Proteus vulgaris*, and 28-220 ppm of bromine was required for *Bacillus spp.* (temperature was not reported). Mold-spores were more susceptible to bromine treatment than were the spores of spore-forming bacteria. Anywhere from 1 to 30 ppm of bromine was required to kill the mold spores in 15 to 30 seconds at room temperature (pH was not reported). Yeasts and non-spore-forming bacteria were the most susceptible to bromine treatment, requiring only about 0.15 mg/L for 15 to 30 seconds at room temperature to achieve "complete kill". The resistance of these classes of organisms to bromine is listed in increasing order: yeasts and non-spore-forming bacteria < mold-spores < spores of spore-forming bacteria.

Goodenough (1964) demonstrated that a bromine residual of 0.8 mg/L could greatly reduce (though not eliminate) total bacterial plate counts in swimming pool water, showing that bromine could be effective at a relatively low concentration. A range of pH values was used in these experiments. Bacterial counts were generally lower at the lowest pH (7.0) and higher at the highest pH (7.8), suggesting that the bactericidal activity of bromine increases as pH decreases, within this pH interval.

Lindley (1966) was able to show that free bromine was highly effective against f2 coliphage and *E. coli* in phosphate buffer (pH 7.5, 0°C). A 4 mg/L dose of bromine was able to achieve about a 4.5 $\log_{10}$ reduction of both f2 phage and *E. coli* within 20 minutes.

Krusé et al. (1970) expanded the work of Lindley (1966) by testing the susceptibility of *E. coli* and f2 phage to bromine treatment at three pH values (6.0, 7.0, and 8.0) at 0°C. At pH 6.0, the approximate percentage concentrations of Br_2 and HOBr are 16 and 84, respectively. At pH 7.0, only HOBr is present. At pH 8.0, the approximate percentage concentrations of HOBr and OBr^- are 85 and 15, respectively. Free bromine achieved 4.7 to 5 $\log_{10}$ reduction of *E. coli* in 10 minutes at pH 6.0 and 7.0, respectively, while only achieving 3.2 $\log_{10}$ reduction at pH 8.0. This suggested that the deprotonated form was less bactericidal than the protonated, which is analogous to the case for free chlorine. For f2 phage, the $\log_{10}$ reductions were 6.5, 3.7, and 2.5 (pH 6.0, 7.0, 8.0, respectively), showing that the same hypothesis held for viral inactivation. The f2 phage seemed to be particularly susceptible to molecular bromine.

Sharp et al. (1975) found that HOBr at a dose of 4.7 μM (0.75 mg/L as Br_2) could inactivate reovirus in phosphate buffered demand-free (PBDF) water at pH 7 and 2°C by more than 3 $\log_{10}$ in 3 minutes (Table 3). Work done by Olivieri et al. (1975) found that 15 mg/L of bromine achieved more than 3 $\log_{10}$ reduction of f2 bacteriophage in PBDF water (pH 7.5, 0°C). The study was designed to elucidate the mode of action of bromine in viral inactivation, and the results suggested that bromine was reacting with the protein portion of the virus (Olivieri et al., 1975).

As part of a larger work investigating the effects of virion aggregation on chemical disinfection kinetics, Floyd et al. (1976) tested the efficacy of free bromine in

the form of HOBr in inactivating monodispersed poliovirus type I in PBDF water at pH 7 and at temperatures ranging from 2 to 20°C. The study showed that initial virus inactivation rates were very high and increased with temperature (Table 3). At 2°C, a dose of 0.21 mg/L (2.2 μM) HOBr inactivated poliovirus by about 1.8 $\log_{10}$ in 16 seconds. As a point of comparison, Scarpino et al. (1972) found that a free chlorine dose around 1 mg/L achieved 2 $\log_{10}$ reduction in poliovirus in 100 seconds (PBDF water, pH 6, 5°C). These data suggest that free bromine may be a stronger virucide than free chlorine at these conditions. The Scarpino et al. (1972) chlorine study did not take the steps, however, to ensure that the virus particles were monodispersed, as was performed by Floyd et al. (1976). A monodispersed stock of virus particles minimizes the occurrence of virus aggregates. Virus aggregates tend to provide a protective barrier against disinfection for virions within the aggregate, and thus slower inactivation would be expected in a non-monodispersed virus stock, such as used in Scarpino et al. (1972). Therefore, a direct comparison between the two studies may not be entirely appropriate. Another chlorine disinfection experiment on monodispersed virus particles would provide more conclusive answers as to which free halogen is the stronger virucide.

Floyd et al. (1978) expanded the aforementioned poliovirus disinfection work to other bromine species, namely hypobromite (OBr^-) and molecular bromine (Br_2). The OBr^- experiments were run at pH 10 and 11 (i.e., above the pK_a of HOBr), and the Br_2 experiments were run at pH 5. Both bromine species were able to achieve around 2 to 4 $\log_{10}$ reductions of poliovirus within 4 seconds at 4 ^oC at concentrations comparable to those used in the previous experiments with HOBr. This finding highlights the ability of free bromine to be an effective virucide over a wide pH range, something that is not true

for free chlorine (Droste, 1997). The mechanism of disinfection by OBr^- has not yet been identified, so it is unclear why the hypobromite ion performs so well compared to the hypochlorite ion.

Free bromine is also an effective bactericide. In a study on the susceptibility of *Pseudomonas aeruginosa* to bromine disinfection, Clark & Smith (1992) indicated that bromine reduced *P. aeruginosa* levels by more than 99% (2 $\log_{10}$) within 10 minutes at 38°C in PBDF water (pH 7.5). These experimental conditions were meant to simulate whirlpool spas, which are known to use a bromine-releasing compound, 1-bromo, 3-chloro, 5, 5-dimethylhydantoin (DiHalo) for disinfection. This highlights bromine's ability to be effective at a high temperature of 38 °C.

Studies performed by Goodenough (1964) showed that at a bromine residual of 0.2 mg/L, an eightfold reduction in total bacterial plate count was observed when the pH of the test water dropped from around 7.7 to 7.0. Similarly, at a residual of 0.8 mg/L, a fivefold reduction in bacterial plate count was seen at the lower pH, suggesting that OBr^- is in fact a weaker disinfectant than HOBr. The finding that OBr- is a weaker disinfectant than HOBr against plate count bacteria is in contrast to the findings of Floyd et al. (1978), that free bromine was highly virucidal against poliovirus virus over a wide pH range from 5 to 11. Further bromine disinfection studies in which both bacteria and viruses are studied under the same bromine disinfection conditions are warranted to help reconcile these conflicting results from separate studies performed under different conditions.

Thomas et al. (1999) found that bromine was able to reduce and control *Legionella pneumophila* levels (both planktonic and sessile forms) about 1 to 3 $\log_{10}$ in

the first few hours in cooling tower waters (pH 7.7, total dissolved solids = 400 ppm) at residuals ranging from 0.5 to 1.5 mg/L as Br_2 (data not shown in Table). However, bromine was ineffective if the residual dropped below 0.5 mg/L because of the depletion of a reactive disinfectant. Quickly disappearing bromine residual is indeed reported elsewhere (Johnson & Overby, 1970; Mercado-Burgos et al., 1975; White, 1998) and is one of the difficulties in using bromine as a disinfectant.

Free bromine is also an effective cysticide against cysts of the protozoan parasite *Entamoeba histolytica*, and much more so than both chlorine and iodine (Stringer et al., 1975). A 3 $\log_{10}$ reduction of cyst viability in phosphate buffered demand free water was achieved in 10 minutes by a 1.5 mg/L dose at pH 4 and a 4 mg/L dose at pH 10. Hence, free bromine maintained its cysticidal efficacy from pH 4 through pH 10; chlorine and iodine are much worse in cysticidal activity at the higher pH values.

2.2.3.2 Bromamine

As explained before, the application of free bromine will invariably result in the formation of bromamines in the presence of nitrogenous substances. Raw sewage almost always has an appreciable ammonia concentration, so bromamine disinfection chemistry and kinetics become very important in wastewater treatment. Recreational waters also contain nitrogenous material as do some drinking sources in certain low resource settings and near coastal areas.

Results of studies in disinfection of microbes in water by bromamine are summarized in Table 4.

Table 4: Inactivation of Microorganisms by Bromamine

Microbe	Water	Halogen Species	Dose	Residual (mg/L^1)	Temp (°C)	pH	Cont. Time	Log_{10} Red.	Reference
E. coli	PB[2]	NH_2Br	0.06 mg/L[3]	N.D.[4]	N.D.	8.2	2 m	~ 0.4	Johannesson, 1958
							10 m	~ 2.4	
							20 m	~ 3.1	
			0.1 mg/L				2 m	~ 1.1	
							5 m	~ 3.1	
							10 m	~ 4.0	
							20 m	~ 4.3	
			0.28 mg/L				1 m	~ 3.1	
							10 m	~ 4.5	
							20 m	~ 5.1	
Total coliform	SWWE[5]	NH_2Br	3.87 mg/L	0.25[6]	10	6.0	20 m	2	Sollo et al., 1975
			2.74 mg/L	0.14	20		20 m	2	
			3.28 mg/L	0.17	30		20 m	2	
		NH_2Br/	2.28 mg/L	0.22	10	7.5	20 m	2	
		$NHBr_2$	2.14 mg/L	0.24	20		20 m	2	
			2.25 mg/L	0.11	30		20 m	2	
		$NHBr_2$	1.64 mg/L	0.11	10	9.0	20 m	2	
			1.14 mg/L	0.08	20		20 m	2	
			1.16 mg/L	0.10	30		20 m	2	
E. coli	TWWE[7]	$NHBr_2$	2 mg/L	0	15	7	1 m	~ 1.1	Johnson & Sun, 1975
			5 mg/L	0.7				~ 2.4	
			10 mg/L	2.8				~ 3.4	
			20 mg/L	6.0				~ 5.0	
		$NHBr_2$	2 mg/L	0	15	7	15 m	~ 0.7	
			5 mg/L	0				~ 2.0	
			10 mg/L	1.5				~ 5.0	
			20 mg/L	5.3				> 5.0	
S. mansoni ovum	SWWE[8]	Bromamine	0.325 M[9]	26.0	N.D.	N.D.	15 m	100%[10]	Mercado-Burgos et al., 1975
			0.300 M	24.0	N.D.	N.D.	30 m	100%	
Polio I	PBDF	NBr_3	3.2 μM	N.D.	4	N.D.	60 s	~ 2	Floyd et al., 1978
			12 μM				16 s	~ 3.2	
			27 μM				12 s	~ 3.1	
			49 μM				12 s	~ 3.2	
Polio I	PBDF	$NHBr_2$	2.9 μM	N.D.	4	N.D.	8 m	~ 3.2	
			13.8 μM				4 m	~ 3.1	
			39 μM				1 m	~ 2.3	

[1] All mg/L measurements are concentrations as Br_2
[2] PB = phosphate buffered water
[3] As Br_2
[4] N.D. = no data
[5] SWWE = secondary treated wastewater effluent
[6] "Average" residual, measured at 10 min
[7] TWWE = tertiary treated wastewater effluent (alum-coagulated and settled trickling filter effluent; 15 mg/L total Kjeldahl nitrogen, 13 mg/L ammonia nitrogen)
[8] SWWE = secondary treated wastewater effluent
[9] Molarity as HOBr
[10] Absence of cercariae, based on observations of 50 to 60 snails

Johannesson (1958) investigated the comparative bactericidal activity of monobromamine and monochloramine. He found that 0.28 mg/L of monobromamine as Br_2 achieved about 3.1 $\log_{10}$ reduction in 1 minute (pH 8.2, temperature was not reported), while the same concentration of monochloramine as Cl_2 achieved less than 0.1 $\log_{10}$ reduction in 15 minutes at the same pH. This "anomalous" behavior of monobromamine and bromamines in general was consistently confirmed in later experiments (below).

Sollo et al. (1975) performed some informative work on the disinfection of wastewater effluents, comparing free bromine and chlorine side-by-side as bactericides. The applied bromine and chlorine were converted to bromamines and chloramines, respectively, as a consequence of the nitrogenous constituents in the wastewater matrix. The brominated effluents consistently had lower levels of total coliforms than the chlorinated effluents. Three pH values were tested: 6.0, 7.5, and 9.0. "Bromine was three times as effective as chlorine at pH 6.0, eight times as effective at pH 7.5, and 30 times as effective as chlorine at pH 9.0" (p. 167). At bromamine doses of 1.14 to 3.87 mg/l, temperatures of 10 to 30^{o}C, and pH levels between 6 and 9, total coliform were reduced by 2 $\log_{10}$ in 20 minutes. The improved efficacy of bromamines with increasing pH was explained by the predomination of the more potent dibromamine species over the monobromamine species at the higher pH. This study pointed out the clear superiority of bromamines over chloramines in bacterial disinfection. Johnson & Sun (1975) indicated further that dibromamine (the predominant bromamine species) was at least twice as effective against *E. coli* as monochloramine (the predominant chloramine species) at equal free halogen mass concentrations in treated secondary wastewater effluent. A 10

mg/L dose of bromamine as Br_2 achieved 5 log_{10} reduction of *E. coli* (pH 7, 15°C) in 15 minutes.

Mercado-Burgos et al. (1975) showed that bromamines in secondary treated wastewater effluent were effective against *Schistosoma mansoni* ova. A residual of about 25 mg/L as Br_2 was able to kill all viable ova in 15 minutes (pH and temperature were not recorded).

Floyd et al. (1978) also investigated bromamine disinfection kinetics of poliovirus. The bromamines were premade with precise ratios of ammonia and free bromine at specified pH values, instead of being produced "in-situ", as in Sollo et al. (1975) and Johnson & Sun (1975). Tribromamine was found to be a stronger virucide than dibromamine, but both were able to reduce poliovirus by more than 3 log_{10} within seconds or minutes of contact time.

2.2.3.3 Chlorine-Bromine Mixtures and Bromine Chloride (BrCl)

Results of studies on the efficacy of chlorine-bromine mixtures and bromine chloride (BrCl) as water and wastewater disinfectants are summarized in Table 5 and Table 6.

Table 5: Inactivation of Microbes by Chlorine-Bromine Mixtures or by Bromine Chloride

Microbe	Water	Halogen Species	Dose	Temp (°C)	pH	Cont. Time	Log_{10} Red.	Reference
E. coli	PB[1]	HOCl	0.75 mg/L	R.T.[2]	5.4	24 h	> 98%[3]	Farkas-Himsley, 1964
						39 h	25%[3]	
						64 h	8%[3]	
		HOBr	0.75 mg/L	R.T.	5.4	24 h	23%[3]	
						39 h	32.5%[3]	
						64 h	3.7%[3]	
		Br:Cl ratio	0.75 mg/L	R.T.	5.4			
	(molar ratios)	75%				24 h	22.8%[3]	
		50%				24 h	23%[3]	
		25%				24 h	33%[3]	
		10%				24 h	> 98%[3]	
						39 h	> 98%[3]	
						64 h	> 98%[3]	
Polio I	PBDF[4]	BrCl	.075 mg/L[5]	25	6	15 m[6]	> 4	Keswick et al., 1978
			.15 mg/L		6	15 m[7]	> 4	
			.15 mg/L		8	15 m[6]	~ 3.6	
			.15 mg/L		9	15 m[6]	~ 3.9	
			.15 mg/L		10	15 m[6]	~ 3.3	
			.05 mg/L		7.5	1 m[6]	0.7	
			.10 mg/L		7.5	5 m[6]	> 4	
			.15 mg/L		7.5	5 m[6]	> 4	
Polio I	PBDF + 1.35 mg/L glycine	BrCl (organic bromamine)	.15 mg/L	25(?)	7.5	15 m	~ 1.1	
	PBDF + 25-100 mg/L NH_4Cl	BrCl (inorganic bromamine)	3 mg/L	25(?)	7.0	15 m	> 4	
Polio I	FASE[8]	BrCl (bromamine)	1 mg/L	25	N.D.	15 m	~ 0.1	
			2 mg/L			15 m	~ 0.4	
			3 mg/L			15 m	~ 1.1	
			5 mg/L			15 m	> 6	
	NFASE[9]	BrCl (bromamine)	1 mg/L	25	N.D.	15 m	~ 0.1	
			3 mg/L			15 m	~ 0.5	
			5 mg/L			15 m	> 5	

[1] PB = phosphate buffer
[2] RT = room temperature
[3] Percent inhibition (not log_{10} reductions), for all Farkas-Himsely (1964)
[4] PBDF = phosphate buffered demand-free water
[5] Measured as HOBr, for all Keswick et al. (1978)
[6] Disinfectant and phosphate buffer pre-mixed for 4 minutes
[7] Disinfectant and phosphate buffer pre-mixed for 10 minutes
[8] FASE = filtered, activated-sludge-treated wastewater effluent (2.4 mg/L organic N, 25.6 mg/L inorganic N)
[9] NFASE = non-filtered, activated-sludge-treated wastewater effluent (18-30 mg/L suspended solids)

Table 6: Inactivation of Microbes by Chlorine-Bromine Mixtures or by Bromine Chloride (cont.)

Microbe	Water	Halogen Species	Dose	Residual (mg/L)	Temp (°C)	pH	Cont. Time	Log_{10} Red.	Reference
f2 phage	NFASE[1]	BrCl	No Data	2.5	15	6	30 m	~ 0.3	Hajenian
				5.7	15	6	30 m	~ 1.3	& Butler,
		HOCl	No Data	2.3	15	6	30 m	~0.3	1980
				4.0	15	6	30 m	~0.6	
Polio I	CDF[2]	BrCl	1.4 μM	No Data	5	5	10 m	~ 3.6	Taylor &
						7	10 m	~ 2.8	Butler,
						9	10 m	~ 2.9	1982
		HOCl	2.8 μM	No Data	5	5	10 m	~ 1.3	
						7	10 m	~ 2.4	
						9	10 m	~ 0.9	
f2 phage	CDF[2]	BrCl	1.4 μM	No Data	5	5	2 m	~ 5.0	
						7	2 m	~ 4.2	
						9	2 m	~ 2.0	
		HOCl	2.8 μM	No Data	5	5	2 m	~ 5.6	
						7	2 m	~ 2.2	
						9	2 m	~ 0.2	
f2 phage	CDF[2] +	BrCl	30 μM	No Data	5	7	2 m	~ 2.0	
	1 mM NH_4Cl	(inorganic				7	5 m	~ 2.4	
		bromamine)				7	10 m	~ 2.5	
	CDF[2] +	HOCl	30 μM	No Data	5	7	2 m	~ 0.3	
	1 mM NH_4Cl	(inorganic				7	5 m	~ 0.3	
		chloramine)				7	10 m	~ 0.3	

Farkas-Himsley (1964) tested the hypothesis that hypobromite and hypochlorite attacked different sites of *E. coli* by looking at any synergistic effects seen in using *mixtures* of hypobromite and hypochlorite together, contrasted with exclusive action of each halogen compound alone. Results from experiments in phosphate buffer at pH 5.4 are shown in Table 5. Five different pH levels were tested, from 5.4 to 8.6, and the following trends were observed at all pH levels. Chlorine alone and the chlorine-bromine mixture (10% bromine by molarity) reduced *E. coli* growth by more than 98% after 24 hours, while bromine alone reduced *E. coli* growth by only 23%. Bromine and chlorine

[1] NFASE = non-filtered activated-sludge-treated wastewater effluent (0.19 mg/L ammonia-N, 18.50 mg/L suspended solids)
[2] CDF = chlorine demand-free water

by themselves did not reduce *E. coli* long-term (up to 64 hours), but the chlorine-bromine mixture maintained its ability to reduce *E. coli* growth.

Additionally, in swimming pool water with high amounts of nitrogenous material at pH 7.2, bromine alone was more effective against *E. coli* than chlorine alone in the long-term. A 0.6 mg/L dose of HOCl resulted in 60% growth in bacteria 18 days after initial halogen exposure, while a 0.6 mg/L dose of HOBr resulted in approximately 99% (2 $\log_{10}$) reduction in bacteria. Though Farkas-Himsely does not explicitly attribute this difference to halamine action, it can be inferred from later studies that halamines are being formed in the presence of nitrogen (Johnson & Sun, 1975; Sollo et al., 1975; Floyd et al., 1978). Therefore, this is another example of bromamines being more bactericidal than chloramines.

Keswick et al. (1978) compared the disinfection efficacy of chlorine and bromine chloride (BrCl) for poliovirus. In phosphate buffer at pH 6, BrCl inactivated 99.99% (4 $\log_{10}$) of the virus after 15 minutes of contact time at a dose of 0.075 mg/L (Table 5), measured as HOBr. Under the same experimental conditions, chlorine achieved the same 4 $\log_{10}$ inactivation at a dose of 0.25 mg/L, measured as HOCl. These results indicated that BrCl was a stronger virucide than free chlorine. The authors also found that dilute solutions of BrCl were less stable than those of chlorine, as evidenced by a larger loss of disinfecting power of BrCl than chlorine when subjected to longer "premixing times" (i.e., the time devoted to mixing the disinfectant into the buffer before the addition of virus). Additionally, the antimicrobial efficacy of BrCl at different pH levels was tested, and BrCl was found to be effective (i.e., greater than 3.5 $\log_{10}$ reduction of virus) over the pH

range of 6-10. This is another advantage that BrCl has over chlorine, because the latter decreases in antimicrobial efficacy above pH 7.5, as noted previously.

Further experiments in buffers modified with the addition of NH_4Cl or glycine (representing inorganic and organic nitrogenous substances, respectively) led to the following observations about bromine: (1) organic bromamines were more virucidal than organic chloramines, and (2) organic bromamines were less virucidal than inorganic bromamines, which is a property that is also evident for chloramines (Krusé et al., 1970). After 15 minutes of contact time, inorganic bromamine reduced poliovirus by >4 $\log_{10}$ but organic bromamine reduced poliovirus by only 1.1 $\log_{10}$. When poliovirus was seeded into both filter-clarified and non-filter-clarified activated sludge wastewater effluent and subjected to various concentrations of BrCl and chlorine, BrCl was superior to chlorine as a sewage disinfectant, and it performed similarly in both filtered and non-filtered sewage effluent with >5 $\log_{10}$ reduction, showing the efficacy of BrCl even in the presence of large particulate organic matter.

Hajenian & Butler (1980) compared the disinfecting power of BrCl and chlorine against f2 coliphage in non-filtered activated-sludge-treated wastewater effluent. The test mixture (15°C, pH 6) was amended to yield a final concentration of 22 mg/L of organic matter. It was found that the presence of organic matter hindered the virucidal activity of chlorine, while BrCl was not as affected. A bromine residual of 5.7 mg/L achieved 1.3 $\log_{10}$ reduction of f2 coliphage in 30 minutes at 15 ^{o}C. Under the same experimental conditions, a chlorine residual of 4.0 mg/L achieved only 0.6 $\log_{10}$ reduction of f2 coliphage in 30 minutes (Table 6).

Taylor & Butler (1982) found bromine chloride to be most active on a molar basis against poliovirus and f2 coliphage among several disinfectants including chlorine in chlorine demand-free water (at 5°C and a range of pH values). At a BrCl dose of 1.4 µM and pH levels between 5 and 9, polio I reductions were 2.8 to 3.6 $\log_{10}$ after 10 minutes and f2 phage reductions were 2 to 5 $\log_{10}$ after 2 minutes at 5 ^{o}C. Under the same experimental conditions, at a HOCl dose of 2.8µM, polio I reductions were 0.9 to 2.4 $\log_{10}$ after 10 minutes, and f2 phage reductions were 0.2 (pH 9) to 5.6 $\log_{10}$ (pH 5) after 2 minutes. With the addition of 1 mM NH_4Cl, the virucidal activity of chlorine was hindered, while BrCl was less affected, just as in Hajenian & Butler (1980). At a BrCl dose of 30 µM f2 phage reductions were 2-2.5 $\log_{10}$ in 2 to 10 minutes at pH 7, while corresponding reductions by HOCl (30 µM dose) were only 0.3 $\log_{10}$.

2.2.4 Summary

Bromine is an extremely versatile disinfectant, as seen by its persistent microbicidal efficacy under varied conditions and chemical speciation against a range of different waterborne microbes of health concern. The literature review shows that bromine can be an effective microbicide over a wide pH range (5 to 11), over a wide temperature range (2 to 38°C), in organic and inorganic nitrogen-containing waters, and in its different chemical forms, including its various bromamine forms. All of these characteristics suggest that bromine is more "robust" than chlorine in a wider range of water quality conditions. However, a major disadvantage of bromine is its high reactivity with ammonia and other substances (Safe Drinking Water Committee, 1980) that could limit its ability to maintain a protective residual.

More direct side-by-side comparisons between bromine and chlorine are needed. Most of the comparisons available in the literature are across different studies and lab groups, where the test microbes and various experimental conditions and protocols are seldom consistent with each other. Given the limited amount of information in the available scientific literature, it is recommended that future studies be done to compare the antimicrobial properties of bromine and chlorine against representative microbes of health concern in drinking water under a variety of experimental conditions.

2.3 DISINFECTION KINETICS

2.3.1 Chick-Watson Model

It has been quite some time now since Harriette Chick (Chick, 1908) first described the inactivation of microorganisms by chemical disinfection as being analogous to a bimolecular elementary chemical reaction:

$$xN + nC \xrightarrow{k_1} pI \tag{1}$$

where N is the active microorganism; C is the disinfectant; k_1 is the reaction rate constant; and I is the inactivated microorganism. Assuming the reaction to be irreversible, the rate law can be written as:

$$\frac{dN}{dt} = -k_1 N^x C^n \tag{2}$$

where x and $n = 1$. If the disinfectant is assumed to be in excess (i.e., C does not change with time), then Equation (2) reduces to Chick's Law:

$$\frac{dN}{dt} = -k^* N \tag{3}$$

where $k^* = k_1 C$ is a pseudo first-order reaction rate constant. Chick's Law states that for a given disinfectant concentration, the rate of microbial inactivation is proportional to the

remaining microbial population. Separation of variables and integration of Chick's Law (with the initial condition that $N = N_0$ at $t = 0$) results in a first-order exponential decay relationship between the survival ratio $\frac{N}{N_0}$ and contact time t:

$$\ln\left(\frac{N}{N_0}\right) = -k^* t \tag{4}$$

2.3.1.1 Chick-Watson Model with Variable Disinfectant Concentration

It is often assumed that chlorine decay is a first-order reaction that is independent of the disinfection reaction (Teefy & Singer, 1990; Lawler & Singer, 1993):

$$C = C_0 e^{-k_{Hal} t} \tag{5}$$

where $C =$ chlorine concentration at time t; $C_0 =$ chlorine concentration at t_0; $k_{Hal} =$ empirically determined halogen decay rate constant; and $t =$ time. When the expression for C is substituted into Equation 2, the following rate equation results:

$$\frac{dN}{dt} = -k_1 * C_0 e^{-k_{Hal} t} N \tag{6}$$

Separation of variables and integration of the above equation yields the following equation:

$$\ln\left(\frac{N}{N_0}\right) = -\left(\frac{k_1 * C_0}{k_{Hal}}\right) * \left(1 - e^{-(k_{Hal} * t)}\right) \tag{7}$$

2.3.2 Deviations from Chick-Watson Model

The Chick-Watson model is often overly simplistic and cannot accurately describe the inactivation of naturally heterogeneous populations of microorganisms. Gyürék & Finch (1998) have developed a comprehensive review of common probabilistic and empirical kinetic models for the disinfection of drinking water. One

such model is called the Hom model. Hom proposed a generalization of the Chick-Watson rate law, in which a new parameter, m, is introduced as follows:

$$\frac{dN}{dt} = -k_1 m N C^n t^{(m-1)} \quad (8)$$

Separation of variables and integration of the equation above yields the Hom model:

$$\ln\left(\frac{N}{N_0}\right) = -k_1 * C * t^m \quad (9)$$

A final model to consider is the One Hit—Two Population (OHTP) model. This theory assumes that the population of target microorganisms is composed of two sub-populations, each with its own first-order decay response to the same chemical disinfection. The formula is as follows:

$$\frac{N}{N_0} = f * e^{(-k_1 * t)} + (1 - f) * e^{(-k_2 * t)} \quad (10)$$

where f is the fraction of the population subject to rate constant k_1, while the quantity $(1 - f)$ is the fraction of the population subject to rate constant k_2.

3 MATERIALS AND METHODS

3.1 MICROBES AND PREPARATION OF MICROBIAL STOCKS

Three microorganisms were used in these experiments as models for major classes of microorganisms and how they might respond to different halogen treatments. The microorganisms, with what they model indicated in parentheses, are: MS-2 coliphages (viruses), *Bacillus atrophaeus* spores (bacterial spores), and *Cryptosporidium parvum* oocysts (protozoan parasites).

3.1.1 MS-2 Coliphages

MS-2 coliphage was used in these experiments as a model for human pathogenic, small, circular RNA viruses like noroviruses, hepatitis A and E viruses, astroviruses and enteroviruses. An MS-2 stock was prepared by infecting an *Escherichia coli* F-amp broth culture in log phase with MS-2 viruses. Viruses were harvested from the infected cell lysates after overnight incubation (16 hours) at 36°C to achieve maximum virus yield. The infected cell lysates were then centrifuged at 4700 x *g* for 20 minutes to remove cell debris, and the virus-containing supernatant was recovered. The supernatant was further purified by chloroform extraction (1:1 volume/volume) and centrifuged at 3500 x *g* for 20 minutes. The supernatant was recovered and dispensed into smaller volumes for storage at -80°C until used for experiments.

On the day of an experiment, a stock volume of MS-2 was thawed and added to levels in test waters to document a 4-$\log_{10}$ (99.99%) reduction, which was an initial

concentration of about 10^5 plaque-forming units per mL of test water. Any excess from the thawed stock was discarded after the experiment. Phosphate buffered saline (PBS) served as the diluent for any intermediate dilution steps, and the volume ratio of diluted stock to test water was 1:100.

3.1.2 *Bacillus atrophaeus* Spores

B. atrophaeus (and other bacterial) spores are often used as indicators of effective kill in routine steam sterilization cycles because of their relatively high resistance to physical and chemical agents. The spores used in experimentation were commercially obtained from SGM Biotech (Bozeman, MT) as a purified spore suspension with a concentration of 2.4 x 10^9 spores/mL. The stock was stored at 4°C and used within six months from the spore manufacture date for the disinfection experiments. Later experiments that studied the effect of the addition of the non-ionic detergent TWEEN® 80 (polyethylene glycol sorbitan monooleate) to the test waters in *B. atrophaeus* spore disinfection experiments were performed no later than seven months after the spore manufacture date. The similarities in disinfection response between the former and latter experiments (as seen in Section 4.2) were sufficient to permit the use of the spore stock even after the manufacturer's suggested expiration date.

The pure spore stock was always vortex mixed for at least five minutes prior to removing aliquots from it with a sterile syringe. Aliquots of the stock were diluted to levels in test waters to document 4-$\log_{10}$ (99.99%) reduction, which was an initial concentration of about 10^5 colony-forming units per mL of test water. PBS served as the diluent for any intermediate spore stock dilutions, and the volume ratio of diluted stock to test water was 1:100. In later experiments where the test waters were amended with

TWEEN® 80 (0.001% v/v), the diluent (PBS) was also supplemented with TWEEN® 80 (0.001% v/v).

3.1.3 *Cryptosporidium parvum* Oocysts

Oocysts of the protozoan parasite, *C. parvum*, Iowa strain, were commercially obtained as a purified suspension from two sources. One batch was received as a purified suspension from Bunch Grass Farm (Deary, ID). Two-day old Holstein bull calves were orally dosed with 2×10^8 oocysts, and the shed stool was collected beginning three days post-dosing. Oocysts were isolated from the stool with diethyl ether, followed by two-stage sucrose gradient (1:4 and 1:2) centrifugation. Next, the oocysts were put over a single layer of cesium chloride and centrifuged. Finally, the purified oocysts were suspended in 50 mL of PBS at a concentration of 2×10^7 oocysts/mL, supplemented with 1000 IU penicillin and 1000 μg streptomycin. The second batch was from the University of Arizona, Department of Veterinary Science and Microbiology (Tuscon, AZ). First, the oocysts were collected from the shed stool of infected calves, then mixed and condensed with 0.1% TWEEN® 80, then purified by differential sucrose and cesium chloride gradients. Finally, the purified oocysts were suspended in 1000 IU penicillin and 1000 μg/L streptomycin at a concentration of 2×10^7 oocysts/mL.

For disinfection experiments, 10 mL aliquots of these stock suspensions were purified by centrifuging at 2600 x g for 15 minutes at 4°C. The supernatant was decanted, and the pellet was resuspended in 10 mL of PBS. Oocyst concentration was enumerated with a hemacytometer under a brightfield microscope. The purified stock suspension was diluted in test waters to a concentration of 1.7×10^5 oocysts per mL of test water to

document a 4-$\log_{10}$ (99.99%) reduction in infectious oocysts. The volume ratio of the purified suspension to test water was 1:100.

3.2 PREPARATION OF REAGENTS AND REACTION VESSELS

3.2.1 Halogen Demand Free Water

Halogen demand free (HDF) water was used in the preparation of all reagents and test waters. The water was made HDF by passing twice-deionized, activated carbon-filtered water through a macroreticular scavenging resin bed (Dracor Corp., Durham, NC).

3.2.2 Halogen Solutions and Measurements

Stock halogen solutions were prepared in amber bottles. Household bleach (6% w/v NaOCl) was diluted with HDF water to a stock concentration of about 500 mg/L as Cl_2. Elemental bromine (Br_2) was purchased from Fisher Scientific (Pittsburgh, PA) and diluted with HDF water to a stock concentration of about 580 mg/L as Br_2.

Halogen concentrations were measured by the DPD colorimetric method with a Hach® Pocket Colorimeter II (Loveland, CO) calibrated for chlorine measurement. The method provided by the colorimeter's instruction manual was followed (Hach, 2009). Chlorine residual concentrations were recorded as measured from the colorimeter, while bromine residuals were recorded as 2.25 times the colorimeter readout (i.e., the ratio of the molecular weight of bromine to that of chlorine). The manufacturer's instruction manual provides a 95% confidence interval of $\pm$ 0.2 mg/L Cl_2 (Hach, 2009) for instrument readouts.

3.2.3 Experimental Waters

HDF water was phosphate-buffered at 0.05M to be used as the experimental test waters. The pH of the water was adjusted drop-wise with 1M NaOH and/or 1M HCl until pH 7.5 was reached.

3.2.4 Reaction Vessels

Sigmacote® (Sigma-Aldrich, St. Louis, MO) was applied to the insides of all reaction vessels and the vials used for *C. parvum* oocyst dilutions to minimize the adherence of the oocysts onto interior walls. This process of applying Sigmacote® was performed once for each container. A small volume (about 10% of the volume of the container being coated) of Sigmacote® was dispensed into the container, the cap was secured onto the container, and the container was vortex-mixed for about one minute. The Sigmacote® was then either transferred to the next container needing a coat or discarded. The coated containers were autoclaved upside down with their caps loosely secured to "seal" the Sigmacote®.

In order to minimize halogen demand exerted by test equipment, all reaction bottles, caps, and stir bars were soaked overnight in a 50 mg/L chlorine bath, then rinsed three times in HDF water prior to use in experimentation.

3.3 PROTOCOL FOR PRELIMINARY HALOGEN DEMAND EXPERIMENTS

A quickly disappearing halogen residual (e.g., within seconds or minutes) would be counterproductive for a disinfection experiment that is supposed to last for several hours. Thus, before proceeding with the disinfection trials, it was necessary to determine that the physical and biological components of the disinfection experiment would not exert an excessive halogen demand.

The objective of these preliminary halogen demand experiments was to measure the halogen demand (if any) of the following variables in separate experiments: Sigmacote®-applied test equipment (physical source of halogen demand); and each of the three microbial stocks (biological source of halogen demand).

Four 125-mL Teflon narrow-mouth bottles (Thermo Scientific, Waltham, MA) were used as reaction vessels. Two bottles were used for each halogen (chlorine and bromine). Of each pair, one bottle contained the variable being tested for halogen demand, and the other served as a control. Each contained a final volume of 100 mL of reaction mixture consisting of: 0.05M phosphate-buffered water, pH 7.5, an aliquot of the microbial stock (if applicable), and halogen. The reaction vessels were placed in a temperature-controlled water bath maintained at 25°C and stirred with a multi-place magnetic stirrer. The reaction mixtures were seeded with enough of the test microorganisms to follow inactivation kinetics over a range of at least 4 $\log_{10}$ (99.99%) reduction (target microbe concentrations listed in Section 3.1). The target halogen concentration in each vessel was 5.0 mg/L (31 μM Br_2, 71 μM Cl_2). Reaction time began with the addition of the halogen. Aliquots (10 mL) were drawn from the reaction vessels at 0 and 1000 minutes, and typically two intermediate time points, and the Hach© colorimeter was used to measure the halogen residual concentrations.

Additionally, small aliquots (2.7 mL) were drawn from the reaction vessels at 0 and 1000 minutes, and quenched with 0.3 mL of sodium thiosulfate (final concentration of 100 mg/L or 0.40 mM), and stored at 4°C until microbial assays (described in Section 3.5) were performed. Although characterizing microbial inactivation over time was not a primary objective in these preliminary halogen demand experiments, the results of the

few microbial assays performed on these samples (not shown) nevertheless served as a helpful reference point for what to expect in the subsequent experiments described in the following section.

3.4 PROTOCOL FOR BATCH DISINFECTION EXPERIMENTS

After verifying sufficiently halogen demand-free test conditions, batch disinfection experiments were performed. The bulk of the experiments were performed in a manner where the microorganisms were tested together in the same reaction vessels, referred to hereafter as the "cocktail" experiments. Further experimentation was done to address organism-specific questions. The protocols for both experiments are given in this section.

3.4.1 "Cocktail" Experiments (Trials i – iv)

Five 125-mL Teflon narrow-mouth bottles (Thermo Scientific) were used as reaction vessels. Two bottles were used for each halogen (chlorine and bromine). Of each pair, one bottle contained the "cocktail" of microorganisms, and the other served as a microbe-negative control. The fifth bottle contained the "cocktail" but not halogen. All vessels contained a final volume of 100 mL of reaction mixture consisting of: 0.05M phosphate-buffered water, pH 7.5, the "cocktail" of test microorganisms (except for the microbe-negative controls), and halogen (except for the halogen-negative control), added in that order. The reaction vessel scheme is summarized in Table 7 and Figure 1.

Table 7: Reaction Vessel Scheme

Reaction Vessel	Chlorine	Bromine	Microbes
A – "MIC only"	–	–	+
B – "Cl, (-) MIC"	+	–	–

C – "Br, (-) MIC"	–	+	–
D – "Cl, (+) MIC"	+	–	+
E – "Br, (+) MIC"	–	+	+

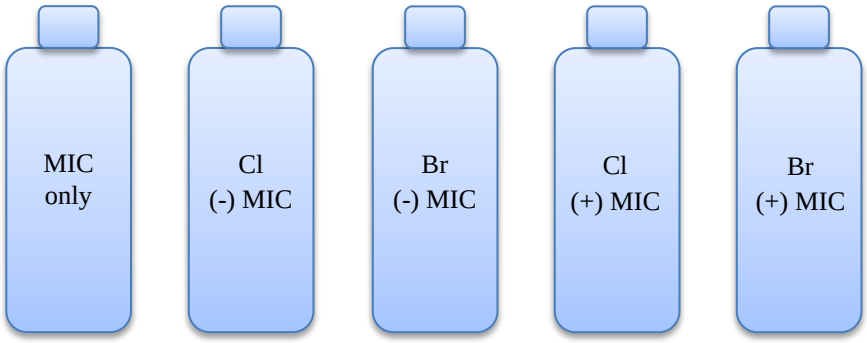

Figure 1: Reaction Vessel Scheme

The reaction vessels were placed in a temperature-controlled water bath maintained at 25°C and stirred with a multi-place magnetic stirrer. The reaction mixtures were seeded with enough of the test microorganisms to follow inactivation kinetics over a range of at least 4 $\log_{10}$ (99.99%) reduction (target microbe concentrations listed in Section 3.1). The target halogen concentration in each vessel was 5.0 mg/L (31 μM Br_2, 71 μM Cl_2), except for the halogen-negative control. Reaction time began with the addition of the halogen. Small aliquots of 2.7 mL were taken from the reaction vessels at typically 0, 1, 10, 30, 100, 300, and 1000 minutes, quenched with 0.3 mL of sodium thiosulfate (final concentration of 100 mg/L or 0.40 mM), and stored at 4°C until microbial assays were performed. Halogen residual concentrations were measured at approximately the same time points. Microbial assays were typically performed within 9, 24, and 12 hours from the last time point for MS-2, *Bacillus* spores, and *C. parvum* oocysts, respectively.

Four such disinfection trials conforming to this protocol were performed, and are hereafter referred to as Trials i through iv. These comprised the major portion of the experimentation presented in this report.

3.4.2 MS-2 High-Titer Experiment (Trial v)

As will be explained in Section 4.2.2, the four disinfection experiments as described above did not yield interpretable data regarding the relative potencies of bromine and chlorine in disinfecting the coliphage MS-2, as MS-2 concentrations were reduced to below detection limits by the first sampling time point (1 minute). In order to obtain comparative data, an additional disinfection experiment (Trial v) was performed using only MS-2, spiked into test water at a higher concentration than in the previous experiments. Aliquots of the virus stock were first subjected to chloroform extraction and centrifugation in order to overcome the higher halogen demand expected from a more concentrated initial MS-2 spike.

After performing a halogen demand experiment in phosphate buffer, it was determined that a ten-fold more concentrated aliquot of diluted MS-2 stock could be used for the purpose of this experiment. The reaction vessel scheme shown in Figure 1 was utilized, using a final reaction mixture volume of 50 mL. Small aliquots were taken from the reaction vessels at 0, 1, 3, 6, 10, and 30 minutes, quenched with sodium thiosulfate and stored at 4°C until the double agar layer plaque assay for virus infectivity was performed the following day.

3.4.3 *B. atrophaeus*, TWEEN® 80, Equimolar Halogen Experiment (Trial vi)

It was determined that equimolar halogen concentrations would provide a useful comparison of the relative disinfecting strengths or potencies of bromine and chlorine,

perhaps more so than using equal mass concentrations. Here, it was chosen to maintain the previous bromine concentration of 5.0 mg/L (31 μM) and to lower the dose of chlorine to about 2.2 mg/L (31 μM). This free chlorine level is more typical of that used in drinking waters, and it would be ill-advised to test a higher concentration of bromine because the World Health Organization gives a guideline maximum value of 5 mg/L for chlorine in drinking water, which was the concentration tested in the aforementioned trials.

These equimolar concentrations were used in an additional experiment on *B. atrophaeus* spore disinfection. It was hypothesized that the spores were aggregating in the reaction vessels of the previous experiments. This phenomenon was to be confirmed by performing *B. atrophaeus* disinfection experiments in the presence and absence of TWEEN® 80, an organic surfactant previously shown to disaggregate or disperse these spores. Several dilutions of TWEEN® 80 were tested to find the most effective concentration in test water that did not concomitantly exert high halogen demand. A final concentration of 0.001% (volume per volume) TWEEN® 80 was most effective in dispersing the spores and was utilized in the equimolar halogen *B. atrophaeus* disinfection experiment solution and in the culture assay dilutions.

3.5 MICROBIAL ASSAYS

The concentrations of the three organisms used in these experiments were determined using their respective assays based on culture or infectivity.

3.5.1 MS-2 Coliphages

Volumes of 100 μL of serially diluted samples from disinfection experiments were assayed by the double agar layer (DAL) plaque technique on the host bacterium, *E.*

coli F-amp using previously described standard procedures (Williams et al., 2001). Plates were incubated aerobically at 36°C for 18 to 24 hours and observed for circular lysis zones (plaques), which were counted. The countable range for pour plate assays is considered to be 30-300 PFU per plate (ASTM D5465-93, 1998). Concentration was expressed as plaque forming units per mL (PFU/mL).

3.5.2 *B. atrophaeus* Spores

Volumes of 100 µL of serially diluted samples from disinfection experiments were spread onto tryptic soy agar (TSA) plates, allowed to dry at room temperature, and incubated aerobically at 36°C for 18 to 24 hours. TSA plates were pre-dried at 36°C for at least 24 hours before the assay was performed to enhance absorption of the sample onto the hardened agar. Orange bacterial colonies that formed were considered to be indicative of *B. atrophaeus* spores, and the concentration was expressed as colony forming units per milliliter (CFU/mL). The countable range for spread plate assays is considered to be 20-200 CFU per plate (ASTM D5465-93, 1998).

3.5.3 *C. parvum* Oocysts

Infectious oocysts were assayed via cell culture infectivity in HCT-8 (human ileocecal colorectal adenocarcinoma) cells, a continuous fast-growing line of epithelial cells considered to be an effective host for *C. parvum* infection (Slifko et al., 1997; Rochelle et al., 2002; Johnson et al., 2012). HCT-8 cells were grown and maintained weekly by serial passage in RPMI 1640 medium (Gibco), supplemented with Fetal Clone 1 bovine serum (10% final concentration, Hyclone), HEPES buffer (15 mM, Mediatech), sodium pyruvate (1 mM, Gibco), and the antibiotics gentamicin and kanamycin. HCT-8 cells were seeded onto eight-well Lab-Tek® II chamber slides (Fisher Scientific) for the

assay. Cells for the assay were observed under a light microscope at a total magnification of 100x, and when the monolayer reached 80% to 90% confluency (typically 48 to 72 hours post-seeding), the medium was aspirated, and 100 μL of the undiluted and 10^{-1} diluted samples (in PBS) from the disinfection experiments were inoculated onto the monolayer. Inoculated cells were incubated at 37^0C (5% CO_2) for 1 hour to allow for initial excystation and infection. Then, 0.5 mL of the modified RPMI 1640 medium was added to each well, and the cells were incubated at 37^0C (5% CO_2) for another 48 hours.

After this incubation period, the culture medium was aspirated, and the cells were fixed with absolute methanol (0.5 mL per well) for 15 minutes at room temperature (RT). The methanol was aspirated, and the cells were washed with PBS (0.5 mL per well) three times. A blocking solution of PBS, supplemented with bovine serum albumin (BSA), was applied for 1 hour at RT (0.5 mL per well). The PBS-BSA was aspirated, and the monolayers were stained with 250-300 μL of purified fluorochrome-labeled monoclonal antibodies (C3C3-FITC) diluted in PBS-BSA, which bind only to the living stages of *C. parvum*. The slides were covered with foil and placed on a shaker platform for 90 minutes at RT. The staining solution was aspirated, and the cells were washed with PBS three times (0.5 mL per well). The chamber walls were removed, cover slips were mounted onto the surface of the slides, and then sealed onto the slides with PVA-DABCO mounting medium.

Cell monolayers on the slides were observed at a total magnification of 250x (oil immersion lens) with the Leitz Orthoplan 2 fluorescent microscope or at 200x (dry lens) with the Olympus BX61 fluorescent microscope. Microscopic viewing fields for each area of cells representing a well on the slide were scored as positive or negative for *C.*

parvum living stages by looking for foci of infection that fluoresced apple-green. Each focus of infection was assumed to come from one infectious oocyst. The Thomas equation was used to calculate a most probable number (MPN) of infectious units (IU) per mL:

$$MPN\ IU/mL = \frac{(number\ of\ positive\ fields)}{[(mL\ sample\ in\ negative\ fields) \times (mL\ sample\ in\ all\ fields)]^{1/2}} \quad (11)$$

3.6 METHODS OF DATA ANALYSIS

3.6.1 Data Output and Error Handling

Halogen residual data are presented as recorded from the Hach© colorimeter readings. In a few instances, a faulty reading was suspected when the initial halogen concentration reading was lower than the following reading, because it is considered not chemically plausible for the halogen to increase in concentration after contacting the test waters. This revealed a possible limitation in the protocol for the first halogen residual measurement for each experiment. On the one hand, the initial measurements would ideally be taken as close to the time of the halogen addition as possible. On the other hand, a certain amount of time would need to elapse to allow for complete mixing of the halogen into the test waters. These conditions resulted in taking the halogen reading typically at around 10 seconds following the halogen addition. The results suggest that, at times, the halogen was not sufficiently mixed when the initial measurement was taken. In these instances, which are specifically identified in Section 4, the initial reading was replaced with the next reading in the time series. Other aberrant cases in which halogen residual increased slightly during an experiment (but not at the beginning) were left

unchanged, as these were not considered to affect total halogen demand calculations or estimates of representative halogen concentrations.

Microbial plate counts of MS-2 and *B. atrophaeus* samples were calculated as indicated in Section 3.5. Only the dilution plates that yielded PFU and CFU counts in the "countable" range (i.e., 30-300 PFU and 20-200 CFU per plate, respectively) were used for analysis. Whenever no dilution plates yielded counts within this range, the two dilution series that yielded counts just above and below the range were used for analysis. Also, whenever a test sample yielded no detectable PFUs or CFUs in the least diluted duplicate plates, the sample was counted as having 1 PFU or CFU on *one* of those plates. This made possible the calculation of a "pseudo" maximum observed $\log_{10}$ microbial reduction in these cases, presented as $> MOLMR$ in the data tables of the Appendix, where $MOLMR$ is the pseudo maximum observed $\log_{10}$ microbial reduction. The actual maximum $\log_{10}$ reduction may in fact be higher, but its determination was not necessary for the scope of this research. Whenever this counting method was applied, the data points in the corresponding figures were noted with "N.D." (not detectable). Infectious oocyst reductions were never large enough to necessitate the use of this method to estimate minimum concentrations and maximum $\log_{10}$ reductions in *C. parvum* data analysis.

3.6.2 Statistical Analyses

Microbial plate counts for MS-2 and *Bacillus* spores were assumed to follow the Poisson distribution, as in Standard Methods (Eaton et al., 2005). Poisson distribution theory states that the expected value, λ, is also the variance. When the count, c, was less than 20, Table 9222:II (Eaton et al., 2005) was utilized to report 95% confidence limits of

the count. For $c \geq 20$, the Poisson distribution closely follows the normal distribution, so the 95% confidence limits were approximated as $c \pm 2\sqrt{c}$, where c represents both the expected value and the variance (i.e., $2\sqrt{c}$ is two times the standard deviation).

Often, it was necessary to perform operations on two values that each had its own estimation parameter. In these instances, error was "propagated", and formulas for the variance of the new parameter are shown below (Ku, 1966). Data analysis required taking the logarithmic survival ratio of separate microbial counts, each of which has their own variance. To compute the 95% confidence limits of the logarithmic survival ratio, the variance of the survival ratio was first calculated with Equation (11), and then the variance of the natural logarithm of the ratio was calculated with Equation (12). The square root of the latter variance (i.e., the standard deviation of the logarithmic survival ratio) was multiplied by two to construct the 95% confidence intervals.

Function form of $\hat{w}$	Approximate formula for variance of $\hat{w}$	
$\frac{\bar{x}}{\bar{y}}$	$\left(\frac{\bar{x}}{\bar{y}}\right)^2 \left(\frac{s_{\bar{x}}^2}{\bar{x}^2} + \frac{s_{\bar{y}}^2}{\bar{y}^2}\right)$	(12)
$\ln \bar{x}$	$\frac{s_{\bar{x}}^2}{\bar{x}^2}$	(13)

The Student's t-Test was utilized to test the null hypothesis that two samples came from the same population with the same mean. Student's t-Tests were performed in Microsoft Excel as two-tailed distributions with matched pairing. The significance level was chosen as $\alpha = 0.05$. Whenever the probability associated with a Student's paired t-Test was less than α, the null hypothesis was rejected.

The JMP software also provided its own statistical output, and the significance of those results is discussed where necessary.

3.6.3 Kinetics Modeling of Microbial Disinfection Data

It was anticipated that the different classes of microorganisms could each exhibit unique behaviors in response to either of the two halogens. As such, a disinfection kinetics model that is effective for the data of one microorganism may not be effective for another. To find a suitable disinfection kinetics model for each test scenario (microorganism and halogen), the simplest model was tested first. If it was deemed a poor predictor of microbial response to the disinfectant, then a layer of complexity was added to the model, and so on.

In the cases where constant disinfectant concentration could be safely assumed, the Chick-Watson model was tested. The natural logarithm of the microbial survival ratio $\frac{N}{N_0}$, where N is the microbe concentration in test water at time t and N_0 is the initial microbe concentration in test water, was plotted against contact time, and a regression analysis was performed in the analytical software, JMP 9.0.0 (SAS Institute, Cary, NC). The rate constants and corresponding standard errors were reported.

In the cases where a constant disinfectant residual could not be assumed, the Chick-Watson model was modified to accommodate the declining residual. It was necessary here to first determine the first-order rate constant for halogen decay (by performing an exponential regression analysis on the halogen residual against contact time in Microsoft Excel), and then to plug the calculated parameters (taken to be constants) into the modified Chick-Watson model created in JMP.

In cases where the modified Chick-Watson model was insufficient, the Hom model was programmed in JMP and used to analyze the microbial survival data. The model parameters and corresponding standard errors were reported. Finally, the One Hit-Two Population model was applied to a few cases. Its model parameters and corresponding standard errors were reported.

4 RESULTS

The results of the preliminary halogen demand experiments are presented first, followed by those of the batch disinfection experiments. Figures and tables have been included where appropriate to illustrate and summarize the findings.

4.1 PRELIMINARY HALOGEN DEMAND EXPERIMENTS

This section presents the results of the preliminary halogen demand experiments performed. Each experiment is summarized by two figures: (1) a line graph comparing the measured halogen residual concentrations in each reaction vessel over contact time and (2) a bar graph comparing the total (i.e., cumulative) halogen demand exerted by the contents of each reaction vessel (including the vessel itself) by the end of the experiment. The figures are all results from single experiments, unless otherwise noted. Replicate halogen measurements and replicate experiments were not considered necessary, as these preliminary experiments were performed just to confirm that the contents of the reaction vessels did not exert an excessive halogen demand.

In the line graphs, the negative control is indicated by open markers, while the presence of the test variable (e.g., Sigmacote® or test microorganism) is indicated by filled markers. Error bars extend 0.2 mg/L above and below each marker, indicating the 95% confidence interval (Section 3.2.2). In the bar graphs, the halogen demand exerted by each reaction vessel is calculated as the difference between the first and last halogen residual reading of an experiment (in the 0 to 10 minute window and typically at the

1,000 minute time point, respectively). Error bars extend 0.28 mg/L[1] above and below each bar, indicating the 95% confidence interval. A brief discussion of the significance of each result is provided.

4.1.1 Sigmacote®

No microorganisms were involved in this experiment. The components possibly exerting a halogen demand in this setup included: the Teflon® reaction bottles, the stir bars, the Sigmacote® application, and the test waters. In Figure 2 are plotted the measured halogen residuals over contact time for vessels in which Sigmacote® was and was not applied. The halogen residual decreased over the 21 hours of the experiment in all reaction vessels by an amount in the range of 0.9 and 1.6 mg/L.

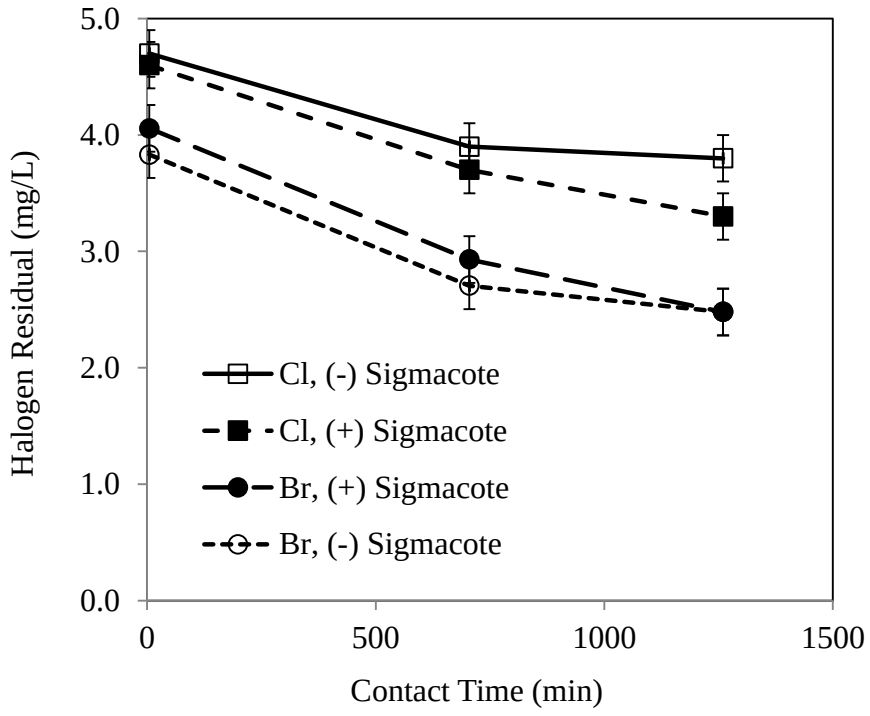

Figure 2: Halogen residual concentrations over time in buffered HDF test water in reaction vessels with and without Sigmacote® treatment

[1] If two random variables are independent, the variance of their difference is the sum of their variances (Ku, 1966).

Figure 3 shows the total halogen demand exerted by the contents of each reaction vessel by the end of the experiment (tf = 21 hours), revealing that the vessels with Sigmacote® application exerted slightly more halogen demand than those without (44% more in the case of chlorine and 17% more in the case of bromine). While Sigmacote® produced a slight difference in halogen demand, the results suggest that most of the halogen demand was exerted by the other test components: the reaction vessels, the stir bars, and/or the test waters.

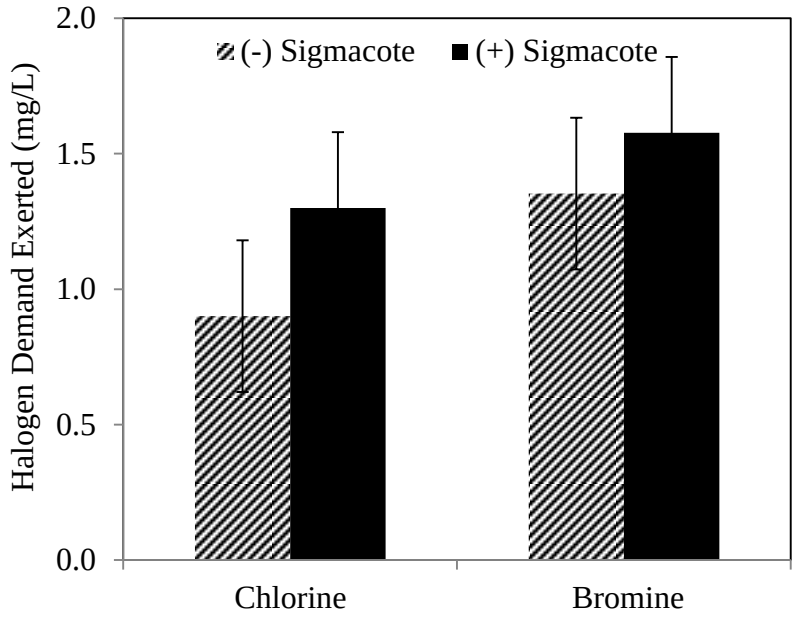

Figure 3: Total halogen demand exerted over a contact time of 21 hrs in test waters of reaction vessels with and without Sigmacote® treatment, relative to initial measured halogen concentration

The application of Sigmacote® to experimental reactors did not appear to exert an excessive halogen demand relative to experimental reactors not treated with Sigmacote®. Therefore, it was determined that Sigmacote® was suitable for application in the Teflon® bottles for subsequent experiments.

4.1.2 Microorganisms

4.1.2.1 MS-2 Coliphages

Next, the reaction vessels (all pre-treated with Sigmacote®) were dosed with MS-2 at an estimated initial concentration of $1.9*10^6$ plaque forming units (PFU) per mL of buffered water. Halogen residual remained relatively stable over time in the presence of MS-2 (Figure 4), indicating that the MS-2 exerted a low halogen demand. In fact, the measured halogen demand in this experiment was lower than that of the Simgacote® experiment, suggesting that the bottles themselves exerted less halogen demand after the first trial run. Based on these results, it was deemed that no further purification steps of the MS-2 were needed.

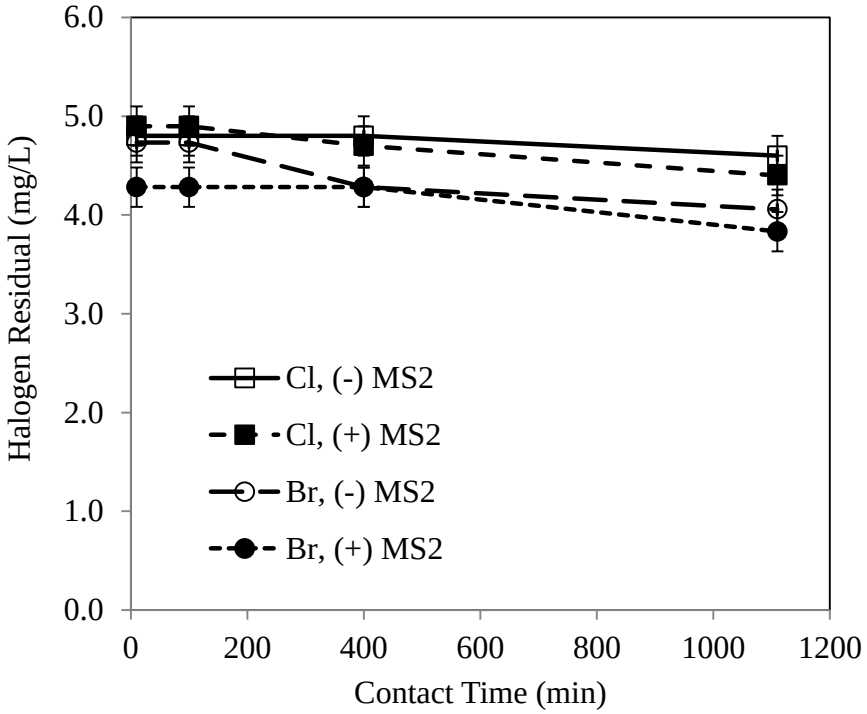

Figure 4: Halogen residual concentrations over time in buffered HDF test water with and without added MS-2

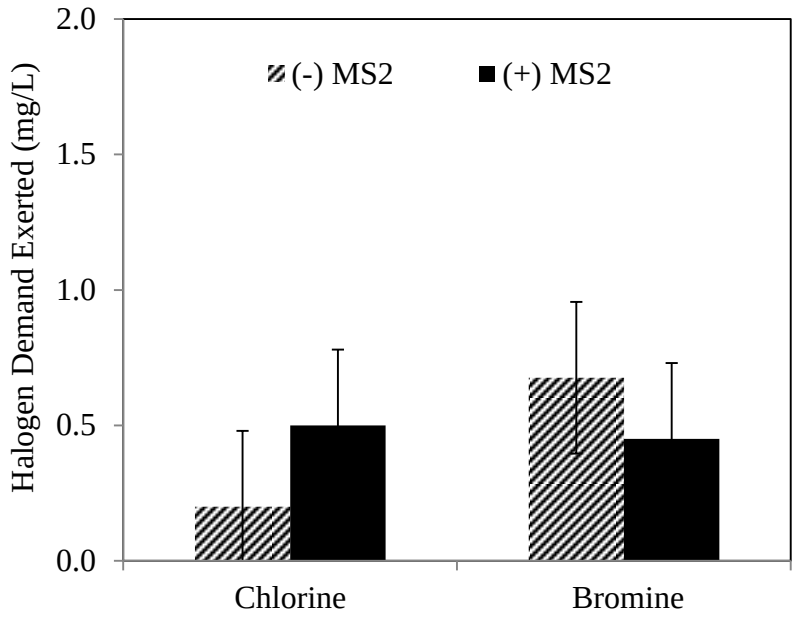

Figure 5: Total halogen demand exerted over a contact time of 18.5 hrs in test waters of reaction vessels with and without added MS-2, relative to initial measured halogen concentration

4.1.2.2 B. atrophaeus *Spores*

The *B. atrophaeus* spores were spiked into buffered HDF test water at an estimated concentration of $1*10^6$ colony forming units (CFU) per mL. As seen in Figure 6 and Figure 7, about 1 mg/L of halogen demand was exerted by the spore stock over a contact time of 1,000 minutes. Based on the relatively small change in halogen concentration in test waters with spores over the duration of the contact time, it was deemed that no further purification steps of spores prior to dosing test water were needed.

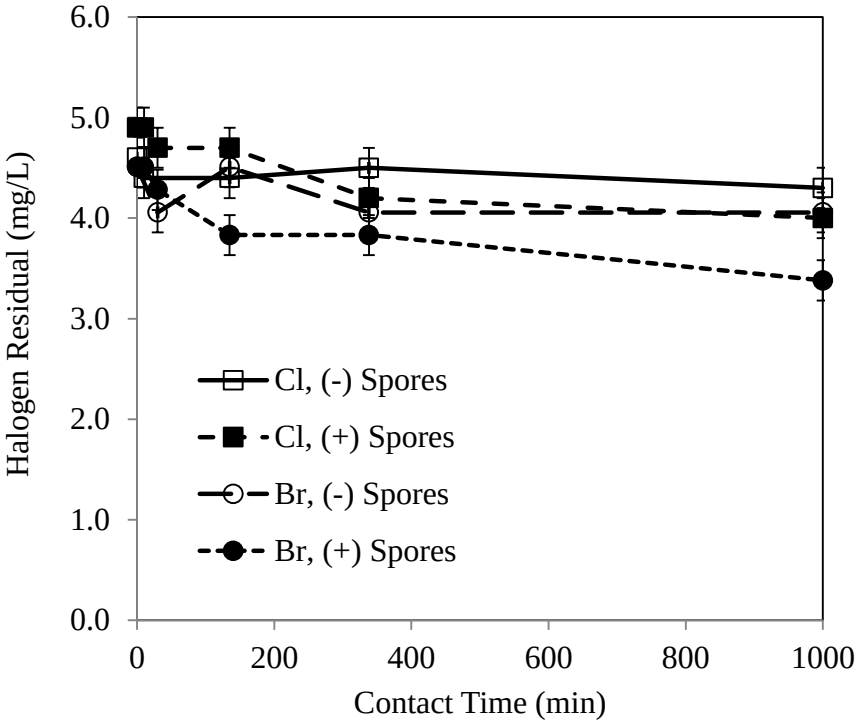

Figure 6: Halogen residual concentrations over time in buffered HDF test water with and without added *B. atrophaeus* spores

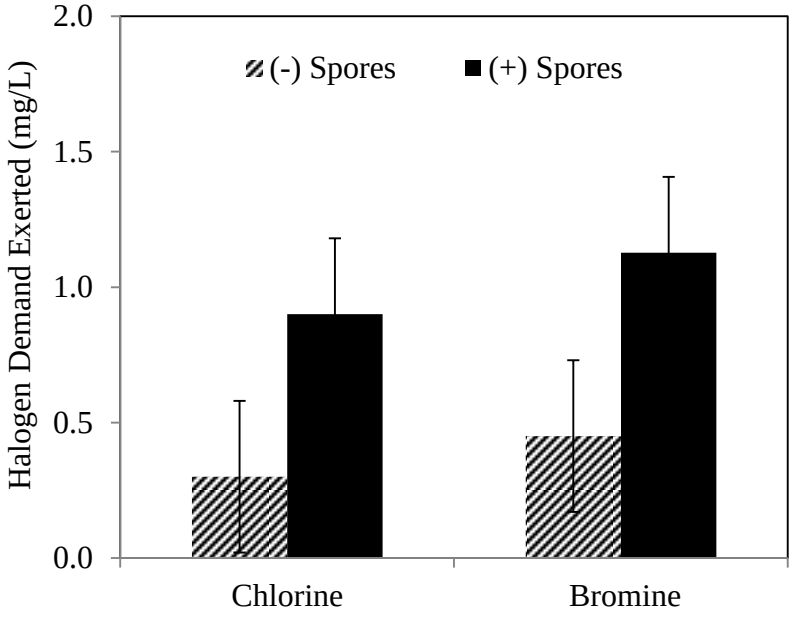

Figure 7: Total halogen demand exerted over a contact time of 16.7 hrs in test waters of reaction vessels with and without added *B. atrophaeus* spores, relative to initial measured halogen concentration

4.1.2.3 C. parvum *Oocysts*

C. parvum oocysts were spiked at an initial estimated concentration of $2*10^5$ oocysts per mL. As seen from Figure 8, *C. parvum* exerted a lot of halogen demand, almost completely depleting the bromine residual at the final time point. Consequently, the oocyst stock was subjected to a further purification step, which was outlined in Section 3.1.3. This further purified stock was then used to perform another halogen demand experiment, the results of which are shown in Figure 9. The further purified oocysts exerted a much lower halogen demand than oocysts that were not further purified (Figure 10). Therefore, the oocysts used for subsequent experimentation always underwent this additional purification step.

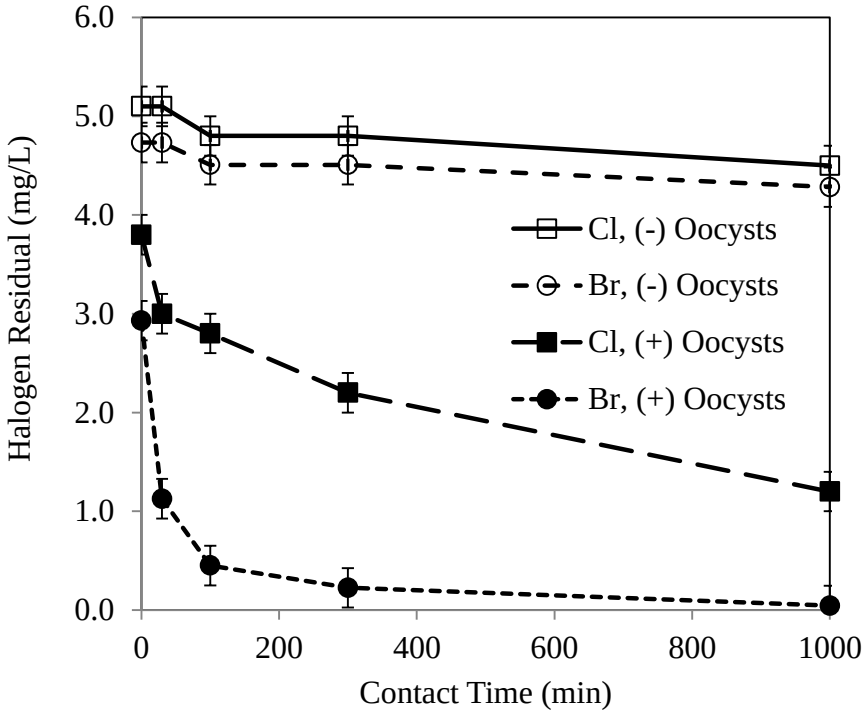

Figure 8: Halogen residual concentrations over time in buffered HDF test water with and without added *C. parvum* oocysts

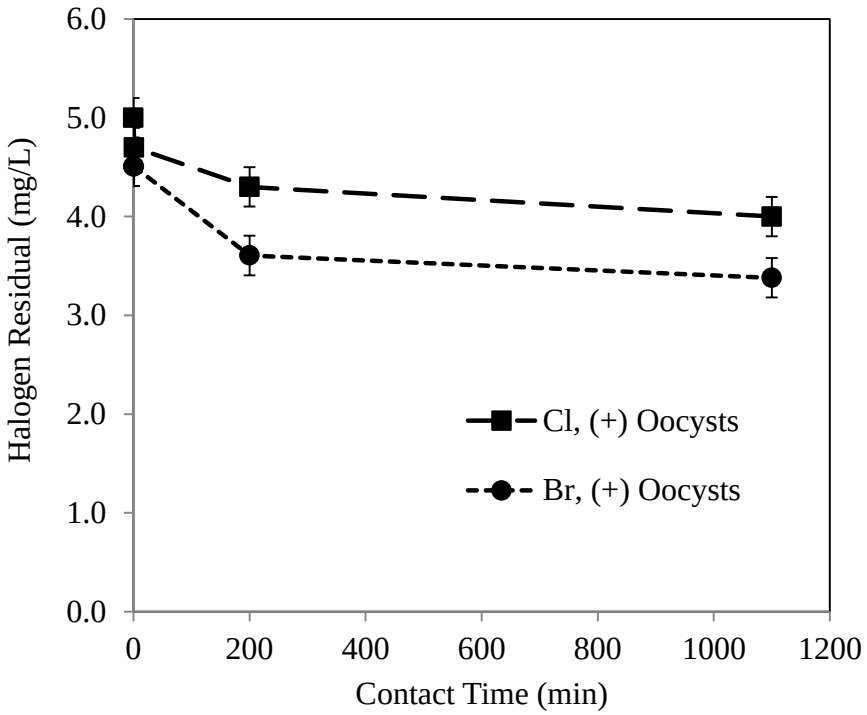

Figure 9: Halogen residual concentrations over time in buffered HDF test water with and without added purified *C. parvum* oocysts

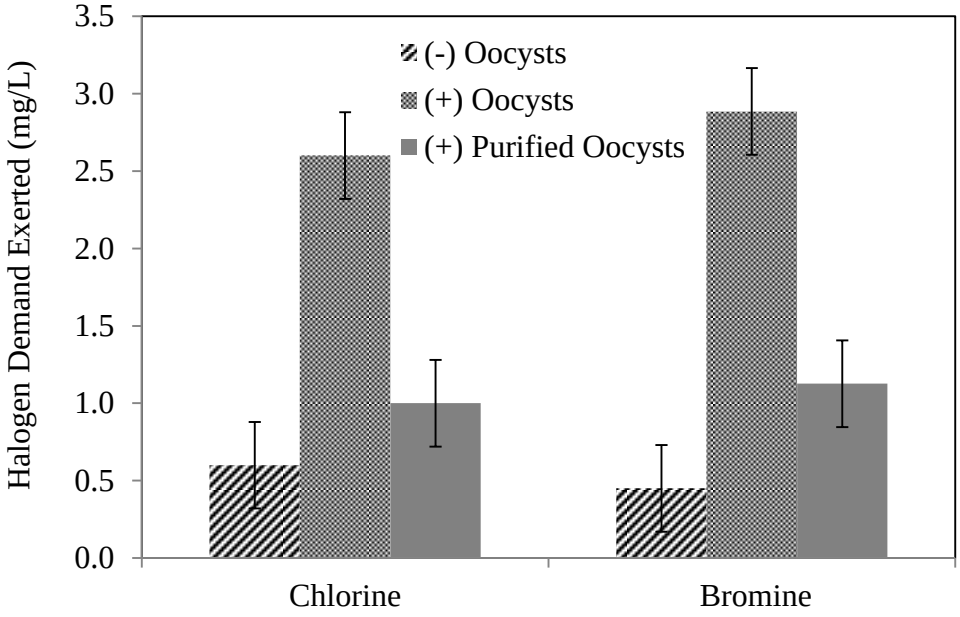

Figure 10: Total halogen demand exerted over a contact time of 16.7 hrs in test waters of reaction vessels with and without added *C. parvum* oocysts (purified and unpurified from stock), relative to initial measured halogen concentration

Figure 11 shows the average halogen demand exerted by the negative controls in all preliminary halogen demand experiments, with the error bars indicating plus and minus two standard deviations (i.e., 95% confidence interval). This is an indication of how much halogen demand may be expected at minimum in future experiments even without the addition of microorganisms.

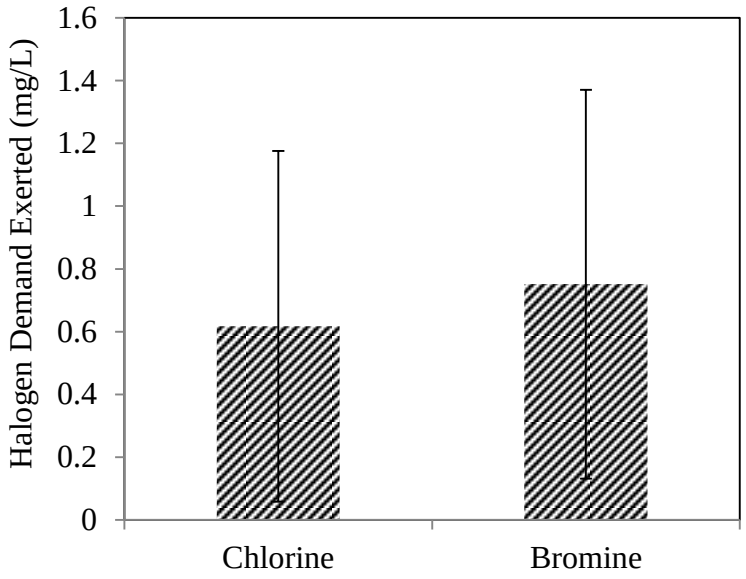

Figure 11: Average halogen demand exerted: All negative controls

4.2 BATCH DISINFECTION EXPERIMENTS

Section 4.1 presented the halogen demand findings of the preliminary experiments. The current section will present the results of the "cocktail" and "special case" disinfection experiments; it begins with some notes on the observed halogen demand, and then separately presents the results for the inactivation of each microorganism by bromine and chlorine disinfection.

4.2.1 Halogen Demand

Halogen residuals decreased over time in all disinfection experiments, indeed conforming to the expectations drawn from the preliminary halogen demand experimental findings in Section 4.1.2. The results from these trials also empirically confirmed the exponential decay nature for both chlorine and bromine. Figure 12 is a plot of halogen residual in each reaction vessel over time for Trial i of the disinfection experiments. The same plots for the remaining trials are located in the Appendix and follow the same trend seen in Figure 12. The open markers indicate single halogen readings from vessels containing no microorganisms, and the closed markers indicate those from microorganism-containing vessels. Error bars represent the manufacturer's 95% confidence interval ($\pm$ 0.2 mg/L) for halogen readings (so throughout for all halogen residual plots). The target initial halogen concentration was 5 mg/L Cl_2 or Br_2 for most experiments, but the observed initial halogen reading was typically between 4 and 5 mg/L, likely a result of a small instantaneous halogen demand (Section 3.6.1) .

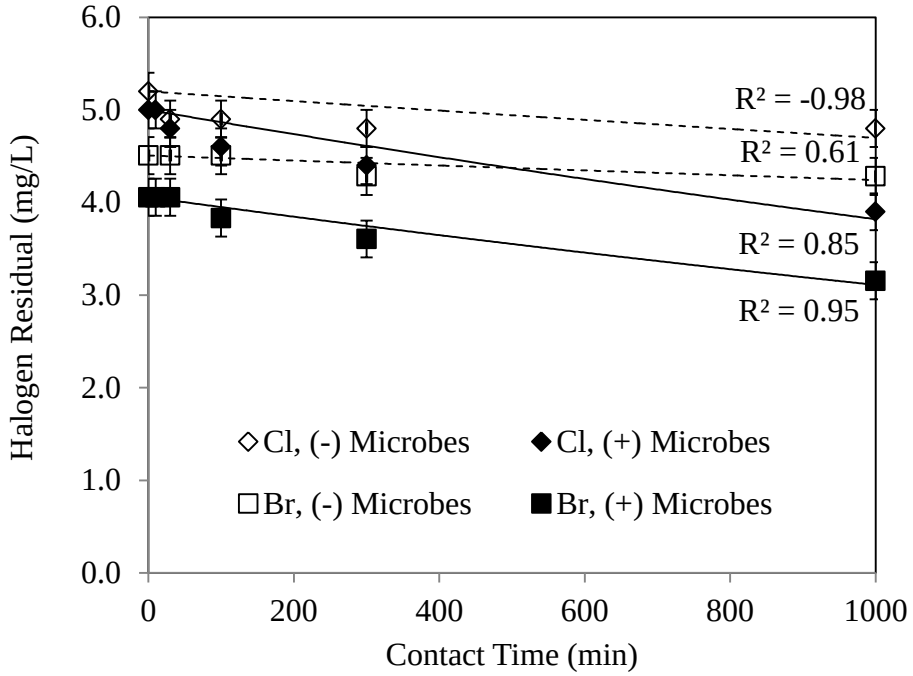

Figure 12: Halogen residual over time in buffered HDF water in reactors with and without test microbes (Trial i)

These data were analyzed by two Student's matched-pair t-Tests. One Student's matched-pair t-Test (two-tailed distribution) compared the array of initial halogen readings with the matched array of final halogen readings for all reaction vessels within each trial and over all trials, to test the null hypothesis that disinfectant concentration remained constant over time. The statistical output is provided in Table 8.

Table 8: Student's matched-pair t-Test comparing initial and final halogen readings for all reaction vessels

Trial	Student's Matched-Pair t-Test p-Value	Statistically Significant at $\alpha = 0.05$?
i	0.050	Yes
ii	0.071	No
iii	0.044	Yes
iv	0.061	No
v	0.084	No
vi	0.006	Yes
All	**0.0001**	**Yes**

The difference between initial and final halogen readings was not statistically significant at the $\alpha = 0.05$ level for three of the six trials, although this was likely due to small sample size. Indeed, when all trials were pooled together, the difference was very significant, so the null hypothesis was rejected.

A second Student's matched-pair t-Test (two-tailed distribution) compared the total halogen demand exerted over the duration of the experiment between the halogen control vessels and the microorganism-containing vessels, to test the null hypothesis that there was no significant difference in halogen demand in test water reactors with and without test microbes. The results of these tests are summarized in Table 9.

Table 9: Student's matched-pair t-Test comparing total halogen demand exerted over the duration of the disinfection experiment by reaction vessels with and without microbes

Trial	Student's Matched-Pair t-Test p-Value	Statistically Significant at $\alpha = 0.05$?
i	0.011	Yes
ii	0.038	Yes
iii	0.038	Yes
iv	0.013	Yes
v	0.178	No
vi	0.038	Yes
All	**0.0005**	**Yes**

In all but one of the trials, the halogen demand exerted in reactors with and without test microbes were significantly different from each other, which means that the cocktail of microorganisms exerted an additional halogen demand above and beyond that exerted by the test equipment and waters.

According to this analysis, any inactivation kinetics models constructed from the data spanning a considerable time portion of the experiment could not hold legitimate claim on the constant disinfectant residual assumption. However, as will be seen in

following subsections, for some experiments it was neither necessary nor useful to consider the data after a certain amount of time because the microorganism was inactivated below detection limits in a short amount of time. In such cases, the data beyond the time point was not incorporated into the model, and so the only data that were used were during the time of the experiment where halogen demand did not decrease too quickly (i.e., a constant halogen residual could be assumed).

In all other cases, the declining residual was incorporated into the kinetic model where possible. The parameters C_0, k_{Cl} (or k_{Br}), and the corresponding R^2 value found for the first-order chlorine and bromine decay reactions for each trial are listed in Table 10 and Table 11, respectively. C_0 was constrained to equal the observed initial halogen reading. The first-order halogen decay constants were used in subsequent inactivation kinetics modeling.

Table 10: First-order rate constants for chlorine decay

Trial	C_0 (mg/L)	k_{Cl} (min^{-1})	R^2
i	5.0	0.00027	0.85
ii	4.4	0.00110	0.99
iii	4.4	0.00064	0.88
iv	4.5	0.00268	0.99
v	4.3	0.00631	0.92
vi	2.4	0.00096	0.90

Table 11: First-order rate constants for bromine decay

Trial	C_0 (mg/L)	k_{Br} (min^{-1})	R^2
i	4.1	0.00027	0.95
ii	4.3	0.00144	0.75
iii	3.6	0.00123	0.90
iv	3.4	0.00509	0.97
v	3.4	0.01670	0.69
vi	5.6	0.00059	0.20

4.2.2 MS-2 Coliphage Inactivation by Bromine and Chlorine

The halogen-quenched samples of the disinfection experiments were assayed for MS-2 survival by the double agar layer plaque method. The survival data of MS-2 are shown in Table 21 through Table 24 in the Appendix. No comparative kinetic data on chlorine and bromine disinfection efficacy could be obtained for MS-2 inactivation in these "cocktail" experiments because MS-2 was completely inactivated to below detection limits very early in the experiments for both halogens (i.e., MS-2 was non-detectable after 1 minute). From these results, it is evident that both bromine and chlorine at an initial dose of 5 mg/L can achieve at least 5-$\log_{10}$ reduction of MS-2 within one minute of contact time (Table 23). Because information on MS-2 inactivation kinetics by bromine and chlorine (pertaining to their relative disinfection efficacies) could not be determined from these experiments, a follow-up MS-2 disinfection experiment was performed with a 10-fold higher initial MS-2 concentration, the results of which are provided in the following subsection.

4.2.2.1 MS-2 Disinfection Kinetics in HDF Buffered Water with 10-fold Higher Initial MS-2 Concentration

A ten-fold higher initial MS-2 seeding density into test water as used in this experiment allowed for better determination and comparison of MS-2 inactivation kinetics by bromine and chlorine in buffered water. The survival data are given in Table 25 of the Appendix and are plotted below in Figure 13. Data points labeled with "N.D." in all microbial survival figures indicate that the organism was "not detected" in the assay and represents the lower detection limit.

Chlorine and bromine were able to inactivate MS-2 beyond the limits of detection (i.e., $>$ 5 $\log_{10}$ reduction); chlorine reduced MS-2 by 5.4 $\log_{10}$ within one minute, and bromine reduced MS-2 by 5.6 $\log_{10}$ within six minutes of contact time. Because the experiment was short in duration, the initial halogen reading was assumed to remain constant.

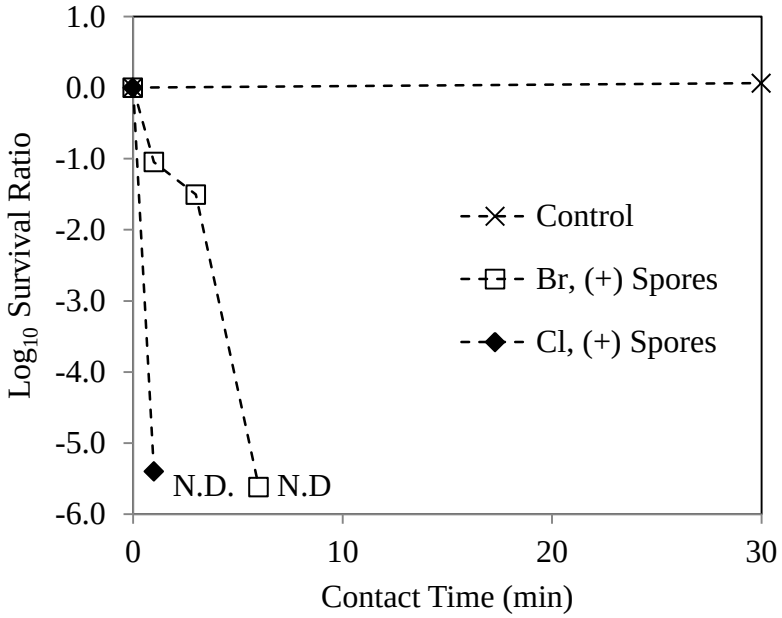

Figure 13: Survival of MS-2 during Disinfection with 5 mg/L Does of Free Bromine and Chlorine in HDF Buffered Water (Trial v)[1]

4.2.2.2 MS-2 Inactivation Kinetics Modeling

Because of the rapid MS-2 inactivation observed in Trials i-iv, a kinetic model could only be applied with data from Trial v. The Chick-Watson model with constant halogen concentration was used. Pseudo first-order MS-2 inactivation rate constants k were determined for chlorine and bromine disinfection by linear regression on a semi-log plot of survival ratio $\left(\frac{N_T}{N_0}\right)$ over time. In step with the Chick-Watson assumptions, the

[1] Data points connected with dotted lines in microbial survival plots such as in Figure 13 were used in kinetics modeling. Data points not connected to the dotted lines (typically non-detect) were not used in kinetics modeling. This paradigm is followed throughout the report for the other microorganisms.

"CT" concept was utilized in order to compare the relative disinfecting strengths of bromine and chlorine. The $CT_{99.99\%}$ (or the product of disinfectant concentration and contact time required to achieve 99.99% (4 $\log_{10}$) reduction) values are shown in Table 12.

Table 12: Chick-Watson model rate constants[1], R^2 values, and CT values for 99.99% (4 $\log_{10}$) reduction of MS-2 by bromine and chlorine

	$k' \pm$ SE [L/(mg*min)]	C_0 [mg/L]	$k \pm$ SE [min^{-1}]	R^2	$CT_{99.99\%}$ [min*mg/L]
Chlorine[2]	2.89 ± 0.00	4.3	12.4 ± 0.00	1.00	< 3.8
Bromine	0.58 ± 0.07	3.4	1.97 ± 0.23	0.92	19.

In the chlorine reaction vessel, MS-2 was still undetectable by the initial 1 minute time point, so the estimated $CT_{99.99\%}$ for MS-2 by chlorine disinfection was less than 3.8 min*mg/L. In the bromine reaction vessel, MS-2 became undetectable by the 6 minute time point. The $CT_{99.99\%}$ estimate for MS-2 by bromine disinfection was calculated to be 19 ± 2.5 min*mg/L, which was five times greater than that of chlorine disinfection. While both halogens were able to achieve greater than 5 $\log_{10}$ MS-2 inactivation in less than 10 minutes, chlorine is more efficacious than bromine as a disinfectant against MS-2 coliphages.

4.2.3 *B. atrophaeus* Spore Inactivation by Bromine and Chlorine

The halogen-quenched samples of the disinfection experiments were assayed for *B. atrophaeus* spore survival by the agar medium spread plate culture method. Data on the survival of *B. atrophaeus* spores disinfected with 5 mg/L doses of free bromine and chlorine in HDF buffered water are presented in Table 26 through Table 29 in the

[1] Note: $(k' * C) = k$
[2] Only two points were available to perform the linear regression; thus, standard errors were zero and R^2=1.0.

Appendix. The results of each of the four trials are presented separately in a semi-log plot in the series of figures below.

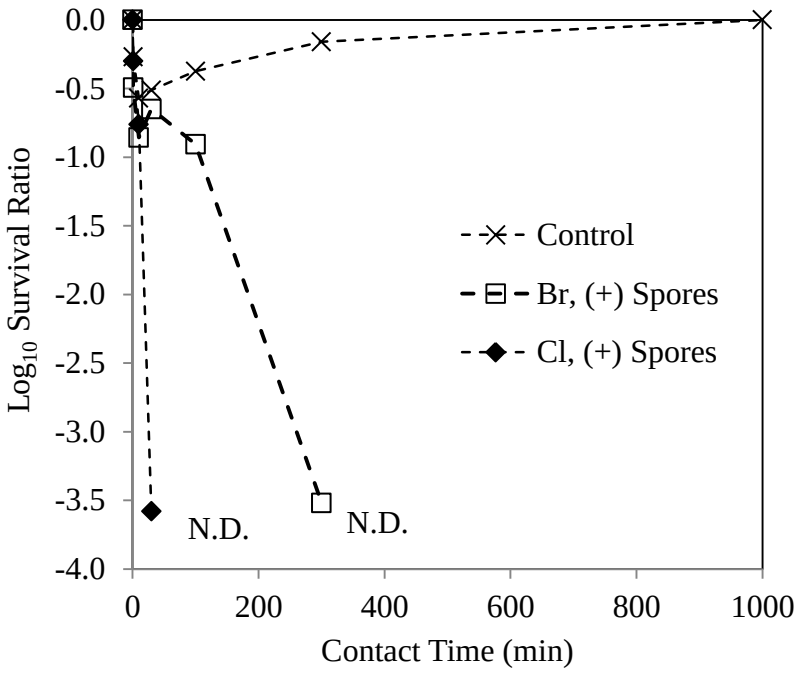

Figure 14: Survival of *B. atrophaeus* spores during disinfection with 5 mg/L doses of free bromine and chlorine in HDF buffered water (Trial i)

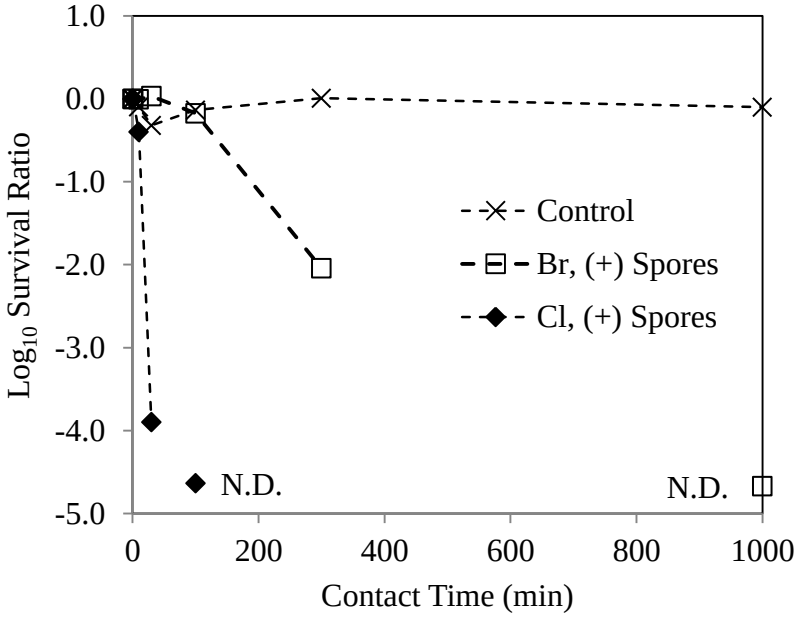

Figure 15: Survival of *B. atrophaeus* spores during disinfection with 5 mg/L doses of free bromine and chlorine in HDF buffered water (Trial ii)

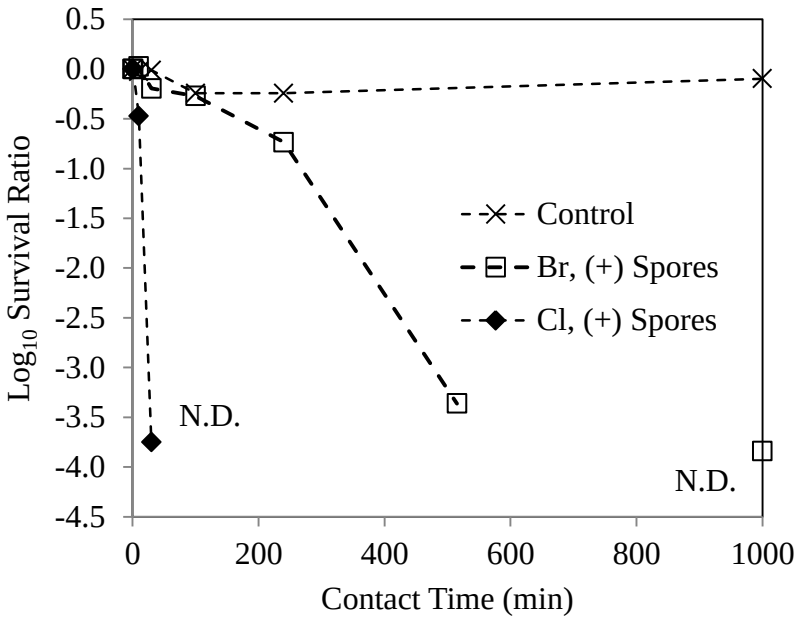

Figure 16: Survival of *B. atrophaeus* spores during disinfection with 5 mg/L doses of free bromine and chlorine in HDF buffered water (Trial iii)

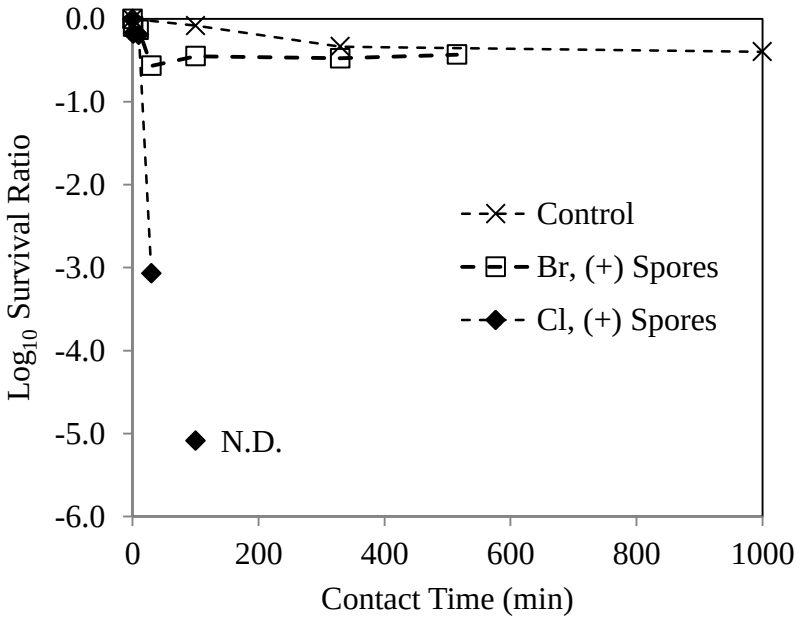

Figure 17: Survival of *B. atrophaeus* spores during disinfection with 5 mg/L doses of free bromine and chlorine in HDF buffered water (Trial iv)

In all trials, the spores persisted longer in the bromine reaction vessels than they did in the chlorine reaction vessels. In Trials i through iii, chlorine disinfection achieved spore reductions of >3.6, 3.9, and >3.7 $\log_{10}$, respectively, in 30 minutes of contact time;

at the same time points, bromine achieved reductions of only 0.7, 0.0, and 0.1 $\log_{10}$. Also, the spores in the chlorine reaction vessels were always reduced to below the assay detection limit (i.e., 3 to 4 $\log_{10}$ reduction) within 100 minutes from the addition of the halogen, while bromine achieved the same reduction levels at typically the 5-hour time point. These results indicate that on this per mass concentration basis, chlorine is the stronger (more efficacious) disinfectant than bromine against *B. atrophaeus* spores.

In Trial iv (Figure 17), bromine achieved 0.6 $\log_{10}$ reduction in spores in 30 minutes, but no further reductions were seen thereafter. Two things may have contributed to this anomalous behavior: (1) a residual too small to exert disinfecting power and/or (2) aggregation of spores. Halogen residual measurements showed that halogen demand was larger in Trial iv (Figure 18 below) than it had been in the three previous trials, and so it is plausible that the smaller residual could have retarded the rate of inactivation or stopped inactivation. The slope of the inactivation curve reached near zero at the 30-minute time point, and at that point the bromine residual was 1.8 mg/L (Figure 18), an amount that seems large enough for bromine to retain its disinfecting power. However, this conjecture could not be confirmed from the previous trials because in the cases where bromine residual dropped to 1.8 mg/L (at around the 600-minute mark in Trials ii and iii, Figure 41 and Figure 42 of the Appendix, respectively), the spores had already been inactivated to non-detectable levels in those trials.

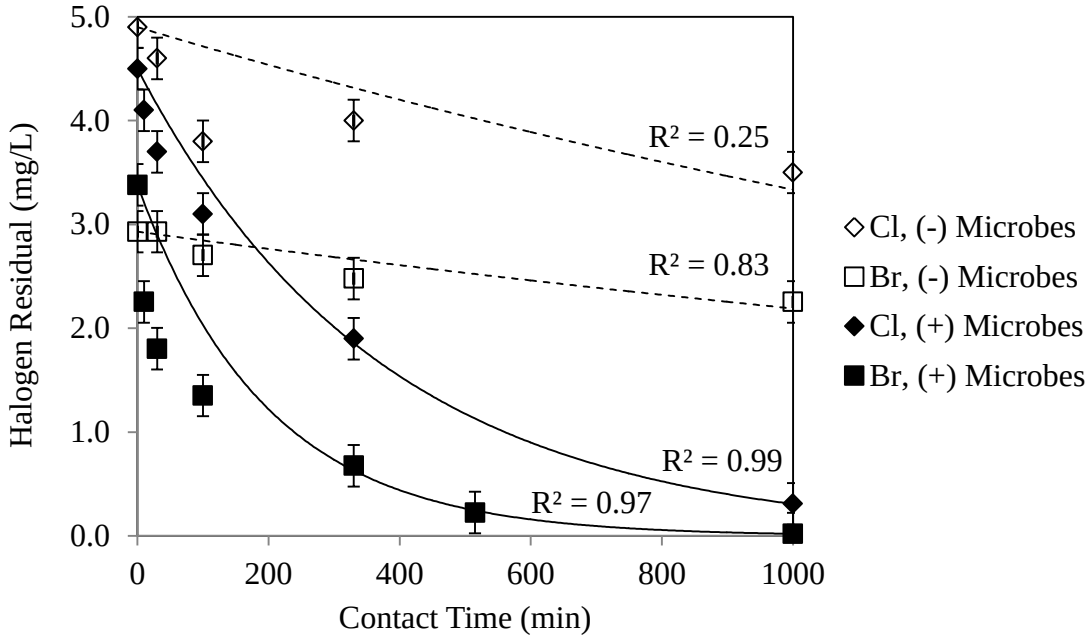

Figure 18: Halogen residual over time in buffered HDF water in reactors with and without test microbes (Trial iv)

An alternative explanation to the persistence of the spores in Trial iv is that the spores had aggregated in the spore stock, and though bromine may have been working effectively to inactivate the spores, the process took longer because of the "protective" aspects of aggregation. The test waters of these four "cocktail" disinfection experiments contained no agent that would prevent spore aggregation, which has been previously recognized to occur in buffered test water (Leech, 2009) such as that used in this study. Therefore, these data probably represent the disinfection kinetics of aggregated spores, which may be the typical physical state of spores in the water environment. In order to minimize *Bacillus* spore aggregation and obtain halogen disinfection data more representative of dispersed spores, an additional experiment was performed in HDF buffered test water containing TWEEN® 80.

4.2.3.1 Disinfection of B. atrophaeus *Spores in Buffered HDF Test Water with TWEEN® 80 by Equimolar Concentrations of Bromine and Chlorine*

The possible effects of aggregation of *Bacillus* spores in the previous experiments were tested indirectly by a simple culture assay experiment of the spore stock. The test waters of three reaction vessels were amended with varying levels of TWEEN® 80 (0.01, 0.001, and 0.0001% final volume/volume), while the test water of a control vessel was unmodified. Equal aliquots of *Bacillus* spores were seeded into each reaction vessel, at an expected final concentration of $1.6*10^6$ spores/mL. No halogens were present in this experiment. The reaction vessels were magnetically stirred for two hours, after which samples were drawn from the vessels for assaying. The results from duplicate plate counts are shown in Figure 19, with error bars indicating 95% confidence limits.

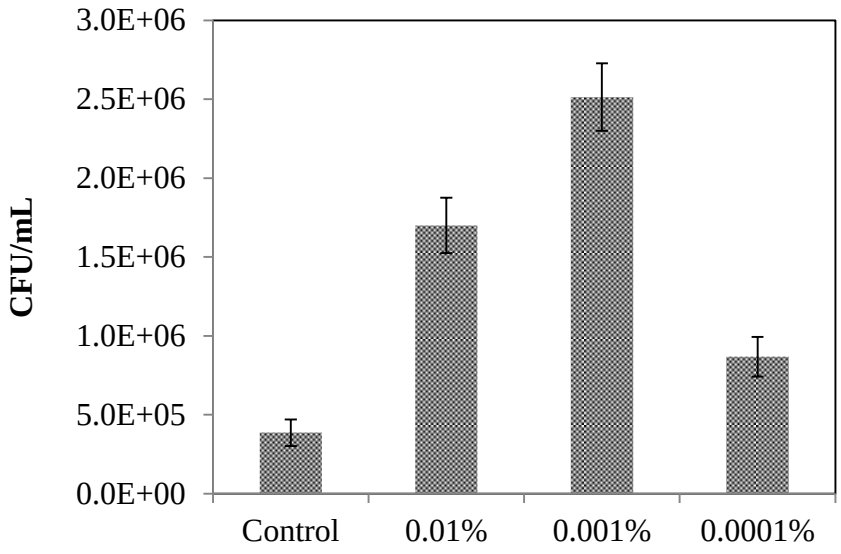

Figure 19: Concentrations of *Bacillus* spores in test water amended with varying levels of TWEEN® 80 (percentage values are final volume/volume) after 2 hrs of contact time

The plate counts from the control vessel yielded the lowest concentration of culturable organisms at $3.9*10^5$ CFU/mL. The reaction vessel amended with 0.001% TWEEN® 80 (final v/v) yielded the highest concentration at $2.5*10^6$ CFU/mL, almost an order of magnitude greater than the control vessel. The experiment confirmed that spores were aggregating in stock and/or upon introduction to the test waters. Therefore, the test

waters for the subsequent equimolar halogen disinfection experiment, "Trial vi", contained 0.001% TWEEN® 80.

The survival data for Trial vi are provided in Table 30 of the Appendix and presented in Figure 20 below. Spores are more rapidly inactivated by chlorine than by bromine. At 100 minutes of contact time, chlorine and bromine reduced spores by 4.3 and 0.2 $\log_{10}$, respectively. These results again suggest that chlorine is the more potent disinfectant of *B. atrophaeus* spores under these experimental conditions, even at equimolar concentrations (31 μM Cl_2 and Br_2; or 2.2 mg/L Cl_2, 5.0 mg/L Br_2).

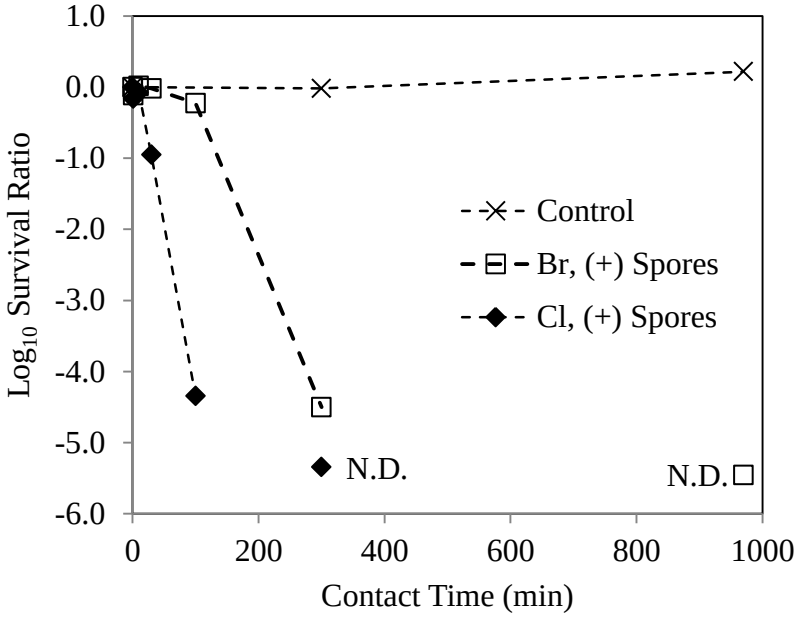

Figure 20: Survival of *B. atrophaeus* spores during disinfection with 5 and 2.2 mg/L doses of free bromine and chlorine, respectively, in buffered HDF water, 0.001% TWEEN® 80 (Trial vi)

4.2.3.2 B. atrophaeus *Spore Inactivation Kinetics Modeling*

Two disinfection kinetic models were tested. First, because halogen concentration was changing for the duration of the disinfection experiments, the Chick-Watson model was modified from its typical constant residual assumption to accommodate a time-dependent residual. As explained in Section 3.6.3, this was

accomplished by replacing the constant C in the Chick-Watson rate law with the expression: $C_0 * e^{-k_{Br(ot\ Cl)}*t}$ before integrating, to obtain logarithmic survival ratio as a function of time and residual concentration. n was assumed to equal one, in the manner of the Chick's Law. The Chick-Watson model formula was as follows:

$$\ln\left(\frac{N}{N_0}\right) = -\left(\frac{k * C_{0,i}}{k_{Cl,i}}\right) * \left(1 - e^{-(k_{Cl,i}*t)}\right) \quad (14)$$

The resulting model parameters, mean squared error, and time to achieve 99% and 99.99% inactivation as predicted by the model are provided for chlorine and bromine disinfection in Table 13 and Table 14, respectively.

Table 13: Modified Chick-Watson model parameters, mean squared error (MSE), and time to achieve 99% and 99.99% *B. atrophaeus* spore inactivation by a 5 mg/L dose of chlorine in buffered HDF water

Trial	k_{Cl} [min^{-1}]	C_0 [mg/L]	$k \pm$ SE[1] [L/(mg*min)]	MSE	$T_{99\%}$ [min]	$T_{99.99\%}$ [min]
i	0.0003	5.0	0.053 ± 0.004	0.36	17	35
ii	0.0011	4.5	0.063 ± 0.008	1.35	16	33
iii	0.0006	4.4	0.060 ± 0.008	1.22	18	35
iv	0.0027	4.5	0.050 ± 0.008	1.17	21	43
vi	0.0010	2.4	0.042 ± 0.003	0.38	46	95

Table 14: Modified Chick-Watson model parameters, mean squared error (MSE), and time to achieve 99% and 99.99% *B. atrophaeus* spore inactivation by a 5 mg/L dose of bromine in buffered HDF water

Trial	k_{Br} [min^{-1}]	C_0 [mg/L]	$k \pm$ SE [L/(mg*min)]	MSE	$T_{99\%}$ [min]	$T_{99.99\%}$ [min]
i	0.0003	4.1	0.0068 ± 0.0008	0.99	169	346
ii	0.0014	4.3	0.0040 ± 0.0006	0.41	340	1,033
iii	0.0012	3.6	0.0046 ± 0.0006	1.09	342	943
iv	0.0051	3.4	0.0021 ± 0.0006	0.27	--	--
vi	0.0006	5.6	0.0060 ± 0.0009	1.98	142	298

[1] SE = standard error. This approximate standard error is computed in JMP as "the product of the root mean squared error and the square root of the diagonals of the derivative cross-products matrix inverse" (SAS Institute Inc., 2010).

	k_{Hal} [min^{-1}]	C_0 [mg/L]	$k \pm$ SE [L/(mg*min)]	MSE	$T_{99\%}$ [min]	$T_{99.99\%}$ [min]
Chlorine	0.0007	4.6	0.059 ± 0.006	0.81	17	34
Bromine	0.0010	4.0	0.0060 ± 0.0009	0.17	299	722

In Trials i-iii, the time required to achieve 99% spore inactivation was 10 to 20 times higher for bromine than for chlorine, and the time required to achieve 99.99% inactivation was 10 to 30 times higher for bromine than for chlorine. Even in Trial vi where the chlorine mass concentration was about half that of bromine (i.e., equimolar), the time to achieve both levels of inactivation was 3 times higher for bromine than for chlorine.

As mentioned previously, the bromine residual decayed rapidly in Trial iv ($k_{Br,iv} = 0.0051\, min^{-1}$, Table 14), severely limiting its disinfecting power. Accordingly, the model could not predict (or extrapolate to) the time to achieve 99% or 99.99% spore inactivation.

The second model tested was the Hom model. As explained in Section 2.3.2, incorporating a declining residual would result in an expression that cannot be integrated to a closed form without advanced calculus functions, which is outside the scope of this report. Instead, the initial halogen reading was assumed to remain constant for the purpose of this analysis. n was assumed to equal one, in order to avoid both over-parameterization and infinitely many solutions. The Hom model formula was as follows:

$$\ln\left(\frac{N}{N_0}\right) = -k * C_0 * t^m \qquad (15)$$

The resulting parameters, mean squared error, and time to achieve 99% and 99.99% inactivation as predicted by the model are provided for chlorine and bromine disinfection in Table 15 and Table 16, respectively.

Table 15: Hom model parameters, mean squared error (MSE), and time to achieve 99% and 99.99% *B. atrophaeus* spore inactivation by a 5 mg/L dose of chlorine in buffered HDF water

Trial	$k \pm$ SE	$m \pm$ SE	C_0 [mg/L]	MSE	$T_{99\%}$ [min]	$T_{99.99\%}$ [min]
i	0.016 ± 0.012	1.37 ± 0.22	5.0	0.19	20	33
ii	0.0017 ± 0.00003	2.07 ± 0.01	4.5	0.00	22	30
iii	0.0017 ± 0.0010	2.07 ± 0.17	4.4	0.03	22	31
iv	0.0003 ± 0.0006	2.50 ± 0.58	4.5	0.08	25	33
vi	0.010 ± 0.005	1.31 ± 0.10	2.4	0.08	55	94

Table 16: Hom model parameters, mean squared error (MSE), and time to achieve 99% and 99.99% *B. atrophaeus* spore inactivation by a 5 mg/L dose of bromine in buffered HDF water

Trial	$k \pm$ SE	$m \pm$ SE	C_0 [mg/L]	MSE	$T_{99\%}$ [min]	$T_{99.99\%}$ [min]
i	0.017 ± 0.024	0.83 ± 0.25	4.1	1.18	156	361
ii	2.6E-6 $\pm$ 1.7E-6	2.27 ± 0.12	4.3	0.003	297	402
iii	7.7E-6 $\pm$ 7.1E-6	2.01 ± 0.15	3.6	0.04	402	568
iv	0.128 ± 0.060	0.16 ± 0.09	3.4	0.10	--	--
vi	3.4E-7 $\pm$ 4.4E-7	2.72 ± 0.23	5.6	0.02	223	288

	$k \pm$ SE	$m \pm$ SE	C_0 [mg/L]	MSE	$T_{99\%}$ [min]	$T_{99.99\%}$ [min]
Chlorine	0.0049 ± 0.0018	1.75 ± 0.11	4.6	0.021	21	31
Bromine	0.0014 ± 0.0020	1.18 ± 0.25	4.0	0.15	295	532

For chlorine disinfection, m values were all greater than one, indicating the presence of an initial "shouldering" in the inactivation kinetics. Similarly for bromine disinfection, m values were greater than one for Trials ii, iii, and vi, in which the Hom model converged on a negligible k value (on the order of 10^{-6} and 10^{-7}), essentially suggesting that contact time was the most important parameter. The implications of this are discussed in Section 5.2. Trial i had a model-predicted m value less than one, suggesting the presence of "tailing off" kinetics, although $m = 1$ is within the window of standard error, $m \pm SE$.

The mean squared error (MSE) was one metric to compare the goodness-of-fit of the respective models with the observed data. The smaller the MSE, the more closely the model resembled the observed data. For spore disinfection by chlorine, the Hom model consistently had a much lower MSE than the modified Chick-Watson model for every trial. For disinfection by bromine, the Hom model again had a lower MSE than the modified Chick-Watson model in all trials except the first. But even then, the modified Chick-Watson model was only slightly more predictive than the Hom (MSE's of 0.99 and 1.18, respectively).

Each figure in the following series overlays the observed data points of each trial with their corresponding inactivation curves as predicted by the modified Chick-Watson and Hom models. The plots visually corroborate that the Hom is the superior model of the two, as the initial shouldering of the observed data points is clearly seen throughout. The modified Chick-Watson formula cannot mathematically model shouldering kinetics. Chlorine disinfection plots are presented first, followed by the bromine disinfection plots.

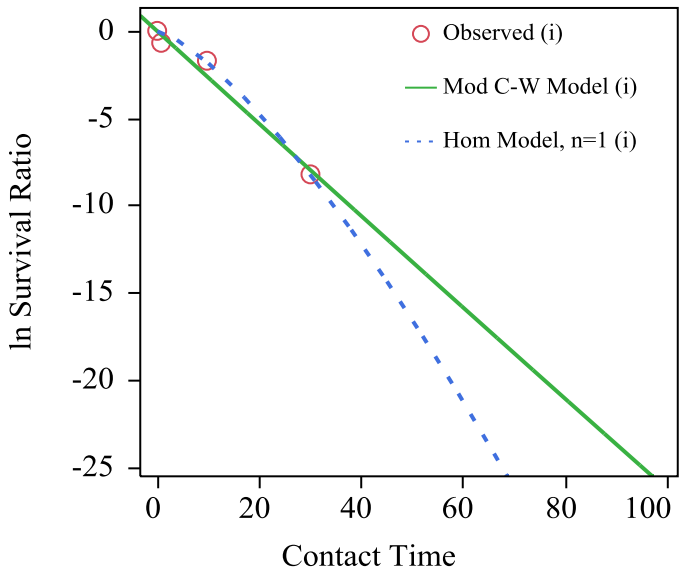

Figure 21: Model-predicted inactivation curves for *B. atrophaeus* spores with 5 mg/L dose of free chlorine in buffered HDF water (Trial i)

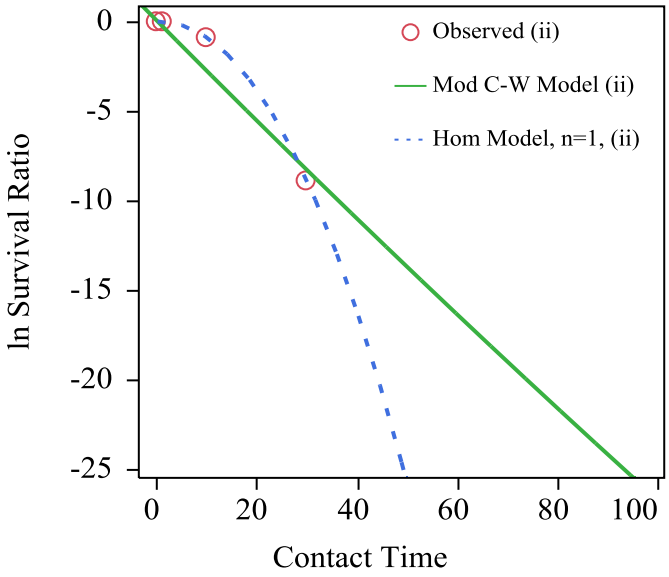

Figure 22: Model-predicted inactivation curves for *B. atrophaeus* spores with 5 mg/L dose of free chlorine in buffered HDF water (Trial ii)

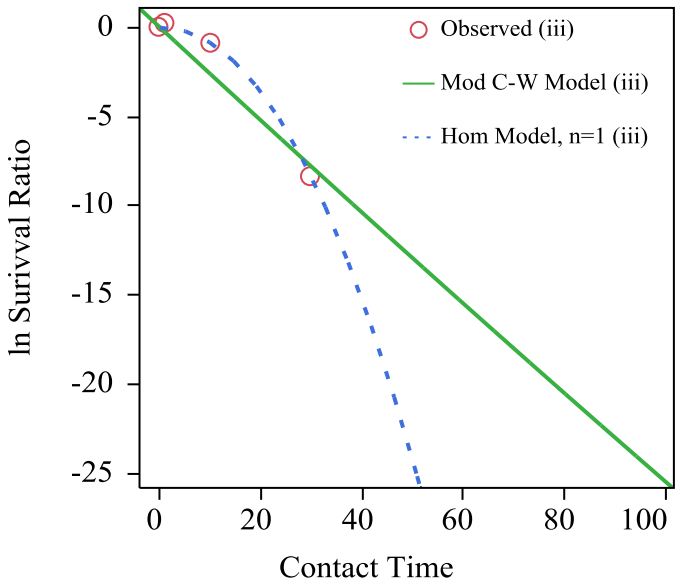

Figure 23: Model-predicted inactivation curves for *B. atrophaeus* spores with 5 mg/L dose of free chlorine in buffered HDF water (Trial iii)

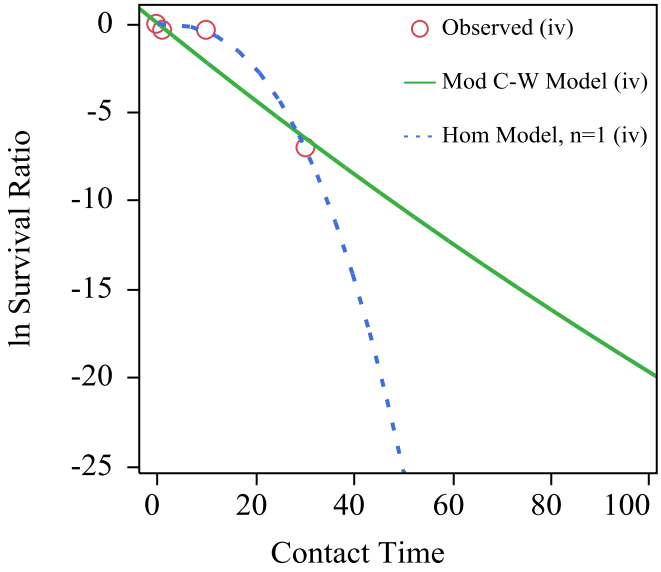

Figure 24: Model-predicted inactivation curves for *B. atrophaeus* spores with 5 mg/L dose of free chlorine in buffered HDF water (Trial iv)

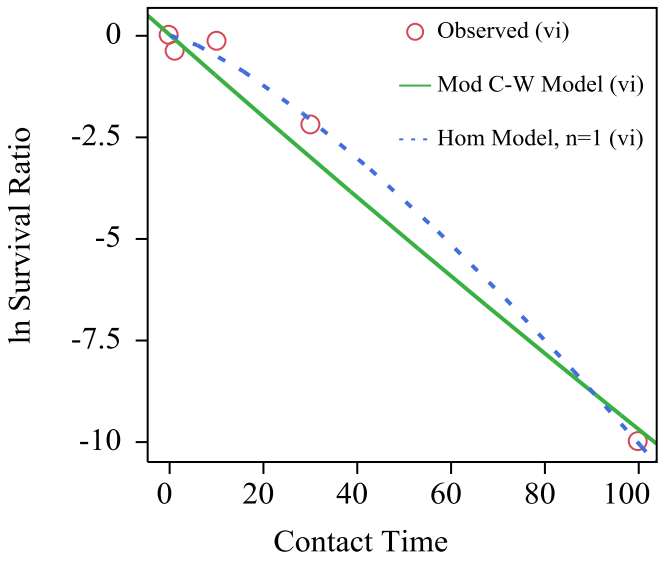

Figure 25: Model-predicted inactivation curves for *B. atrophaeus* spores with 2.4 mg/L dose of free chlorine in buffered HDF water (Trial vi)

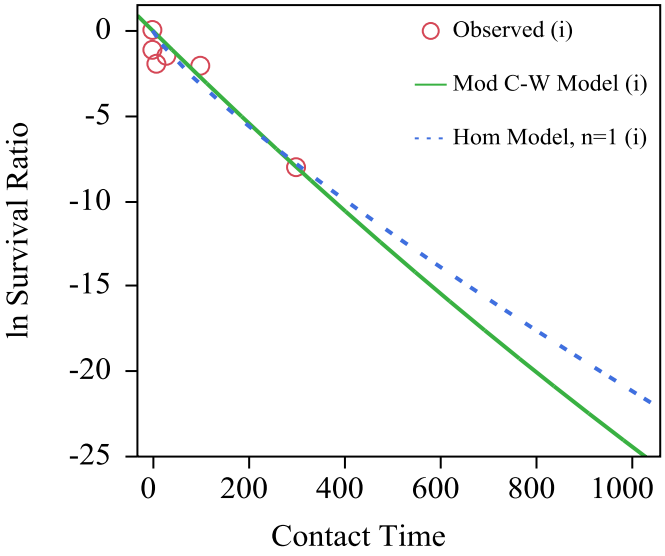

Figure 26: Model-predicted inactivation curves for *B. atrophaeus* spores with 5 mg/L dose of free bromine in buffered HDF water (Trial i)

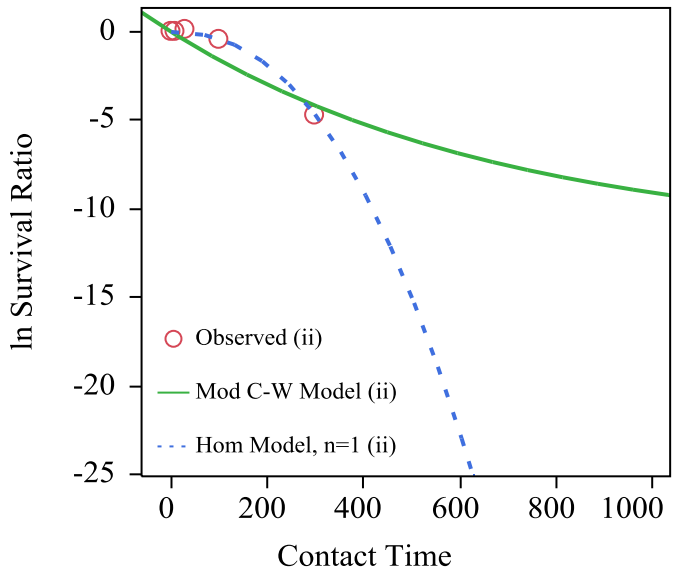

Figure 27: Model-predicted inactivation curves for *B. atrophaeus* spores with 5 mg/L dose of free bromine in buffered HDF water (Trial ii)

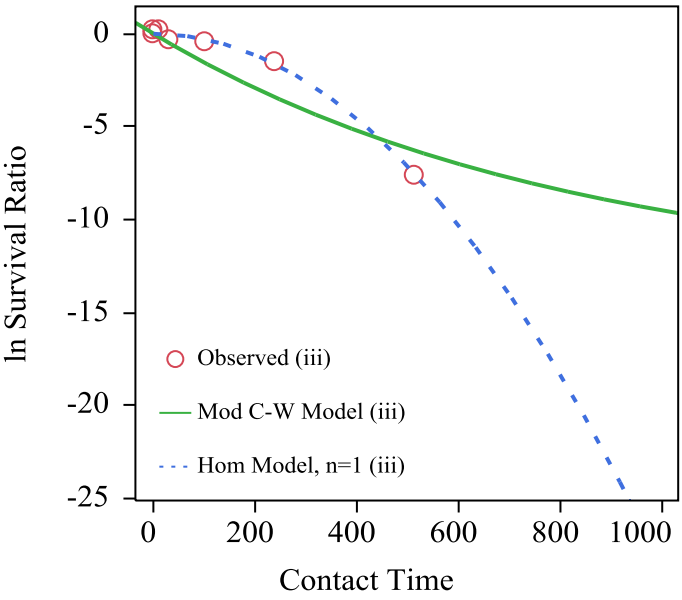

Figure 28: Model-predicted inactivation curves for *B. atrophaeus* spores with 5 mg/L dose of free bromine in buffered HDF water (Trial iii)

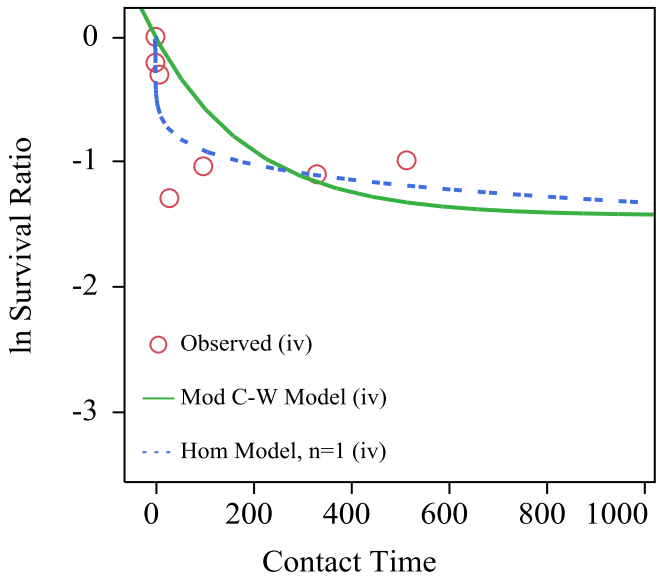

Figure 29: Model-predicted inactivation curves for *B. atrophaeus* spores with 5 mg/L dose of free bromine in buffered HDF water (Trial iv)

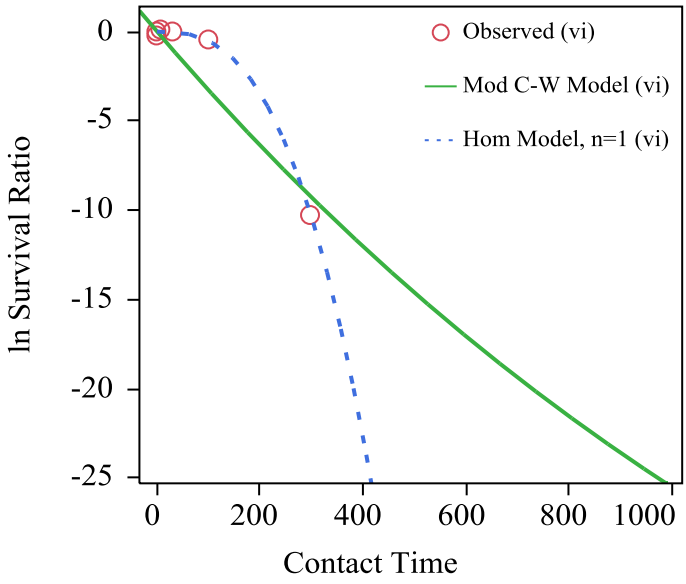

Figure 30: Model-predicted inactivation curves for *B. atrophaeus* spores with 5 mg/L dose of free bromine in buffered HDF water (Trial vi)

4.2.4 *C. parvum* Oocyst Inactivation by Bromine and Chlorine

The halogen-quenched samples of *C. parvum* oocysts in test waters that were subjected to 5 mg/L doses of free bromine and chlorine were inoculated onto HCT-8 cell

monolayers in slide wells for the infectivity assay. The results from all four *C. parvum* oocyst halogen disinfection experiments are shown in Figure 31 as infectious *C. parvum* oocyst concentrations in test water on a logarithmic scale plotted against contact time. A microscopic image of a typical focus of fluorescent antibody-labeled living stages of *C. parvum* in infected HCT-8 cells is shown in Figure 32.

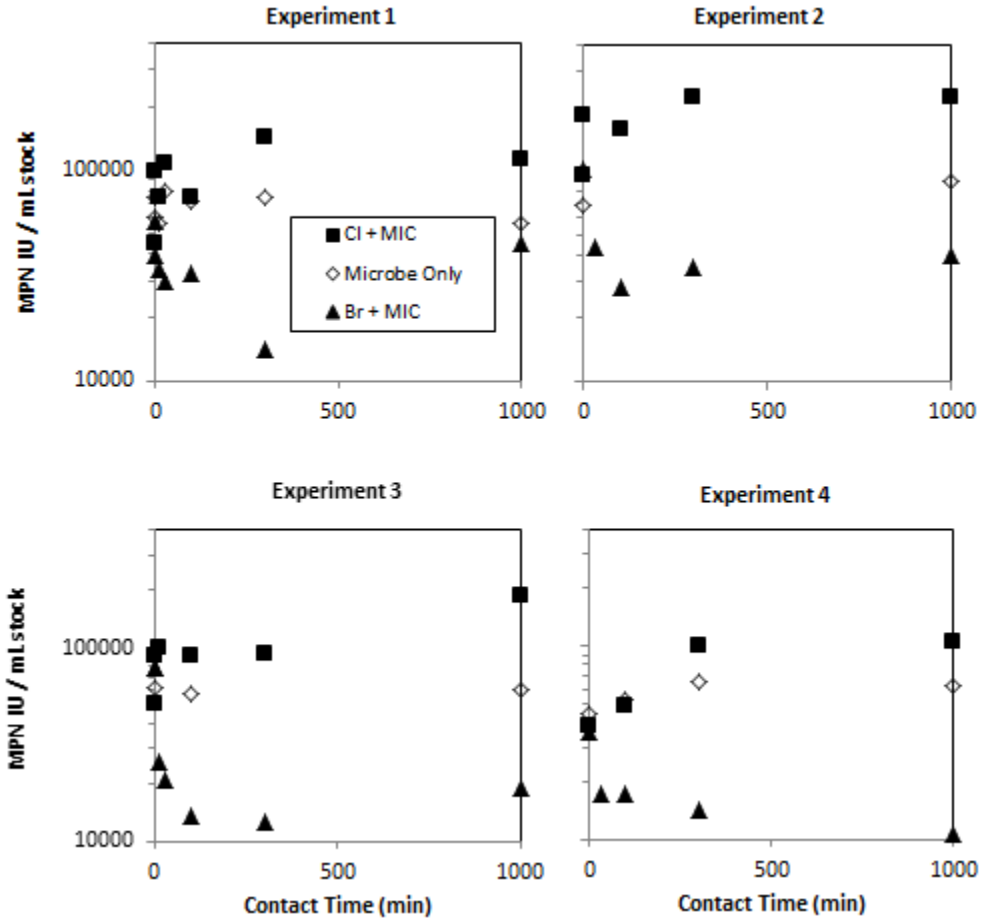

Figure 31: *C. parvum* oocyst infectivity over time in buffered HDF test water disinfected with a 5 mg/L dose of free bromine or chlorine (Trials i-iv)

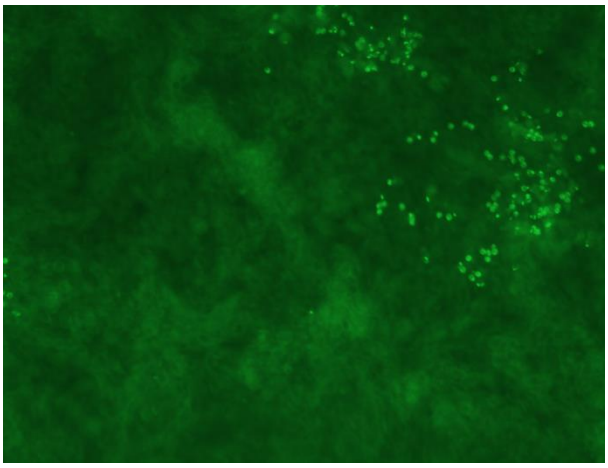

Figure 32: Fluorscent focus of antibody-labeled *C. parvum* living stages in infected HCT-8 cells

According to the results shown in Figure 31, some overall observations can be made about the changes in infectivity of *C. parvum* oocysts with respect to exposure to 5 mg/L doses of each halogen in buffered test water over time. First, it is apparent that treating oocysts with 5 mg/L chlorine in pH 7.5 buffered water actually enhanced the excystation and infectivity of *C. parvum* oocysts in the cell culture assay system used. In contrast to the disinfection results for chlorine, 5 mg/L bromine disinfection in the same test water and at the same test conditions does not exhibit the *C. parvum* oocyst infectivity-enhancing phenomenon observed with chlorine disinfection. In contrast to chlorine, the 5 mg/L dose of bromine reduces the concentration of infectious *C. parvum* oocysts.

4.2.4.1 C. parvum *Oocyst Inactivation Kinetics Modeling*

All four experimental trials confirm the same general pattern of results, which are an apparent increase in *C. parvum* oocyst infectivity upon exposure to a 5 mg/L dose of free chlorine and a consistent decrease in *C. parvum* oocyst infectivity upon exposure to a 5 mg/L dose of free bromine. The decrease in *C. parvum* oocyst infectivity by bromine

over time appears to follow retardant or "tailing off" inactivation kinetics, with a plateau (approach to 0 slope for the rate of inactivation) at about 0.5 to 1 log10 reduction, a major departure from first-order disinfection kinetics.

Two kinetic models were tested for chlorine "disinfection". First, because chlorine concentration was changing for the duration of the disinfection experiments, the modified Chick-Watson model was used. The resulting model parameters, mean squared error, and time to achieve a 2- and 4-fold increase in infectivity as predicted by the model are provided in Table 17.

Table 17: Modified Chick-Watson model parameters, mean squared error (MSE), and time to achieve a 2- and 4-fold increase in *C. parvum* oocyst infectivity by a 5 mg/L dose of chlorine in buffered HDF water

Trial	k_{Cl} [min^{-1}]	C_0 [mg/L]	$k \pm$ SE [L/(mg*min)]	MSE	$T_{200\%}$ [min]	$T_{400\%}$ [min]
i	0.0003	5.0	-0.00028 $\pm$ 0.00015	0.51	532	1,152
ii	0.0011	4.5	-0.00031 $\pm$ 0.00016	0.23	713	--
iii	0.0006	4.4	-0.00050 $\pm$ 0.00016	0.30	348	798
iv	0.0027	4.5	-0.00081 $\pm$ 0.00032	0.36	264	--

k_{Cl} [min^{-1}]	C_0 [mg/L]	$k \pm$ SE [L/(mg*min)]	MSE
0.0007	4.6	-0.0003 $\pm$ 0.0001	0.19

Because infectivity increased from the chlorine treatment, the k values in Table 17 are negative.

The Hom model (with $n = 1$) was also applied to the chlorine "disinfection" of *C. parvum* oocysts. The resulting parameters, mean squared error, and time to achieve a 2-fold increase in infectivity as predicted by the model are provided in Table 18.

Table 18: Hom model parameters, mean squared error (MSE), and time to achieve a 2-fold increase in *C. parvum* oocyst infectivity by a 5 mg/L dose of chlorine in buffered HDF water

Trial	$k \pm$ SE	$m \pm$ SE	C_0 [mg/L]	MSE	$T_{200\%}$ [min]
i	-0.138 $\pm$ 0.053	0.046 $\pm$ 0.081	5.0	0.13	1.1
ii	-0.152 $\pm$ 0.031	0.000 $\pm$ 0.040	4.5	0.02	0.0
iii	-0.134 $\pm$ 0.043	0.100 $\pm$ 0.060	4.4	0.07	5.0
iv	-0.193 $\pm$ 0.104	0.003 $\pm$ 0.106	4.5	0.24	0.0

$k \pm$ SE	$m \pm$ SE	C_0 [mg/L]	MSE
-0.117 $\pm$ 0.024	0.032 $\pm$ 0.042	4.6	0.02

According to the MSE metric, the Hom model is a better predictor of the infectivity increase by chlorine than the modified Chick-Watson model. The time to achieve 2-fold increase in infectivity is virtually zero or close to zero (Table 18). This is, in fact, a good representation of the behavior of the observed data, which shows a sharp increase in infectivity near the beginning of the experiment.

Each figure in the following series overlays the observed data points of each trial with their corresponding inactivation curves as predicted by the modified Chick-Watson and Hom models. Visually, neither model is a great fit to the data, but the Hom would certainly be preferable to the modified Chick-Watson model, which is especially poor at predicting behavior near the origin. The Hom model captures well the sharp increase in infectivity near the origin.

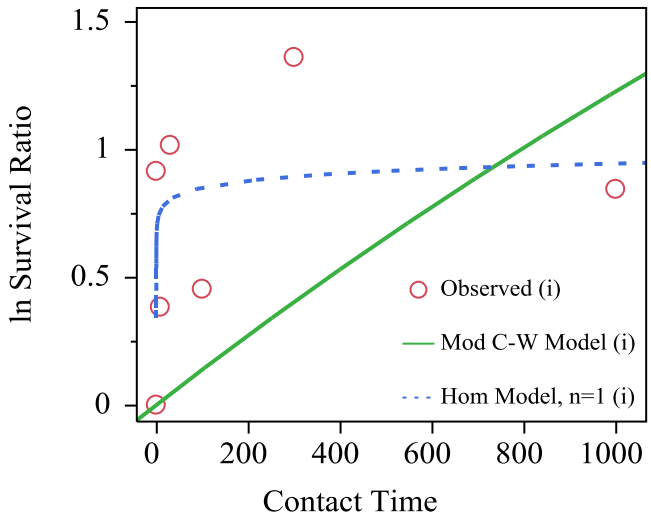

Figure 33: Model-predicted inactivation curves for *C. parvum* oocysts with 5 mg/L dose of free chlorine in buffered HDF water (Trial i)

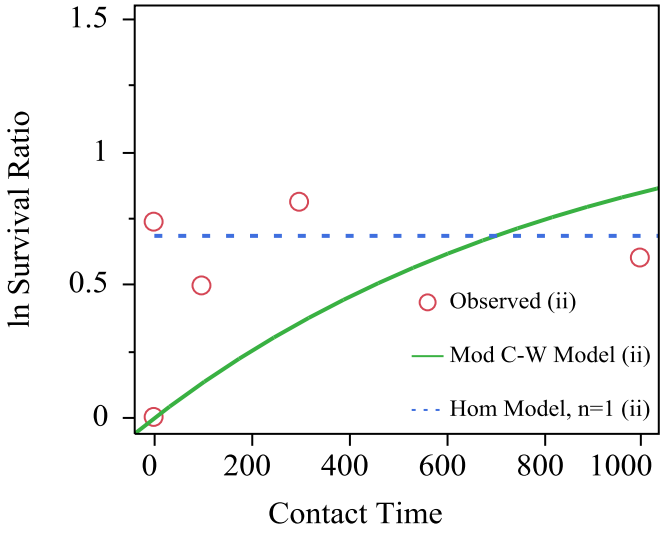

Figure 34: Model-predicted inactivation curves for *C. parvum* oocysts with 5 mg/L dose of free chlorine in buffered HDF water (Trial ii)

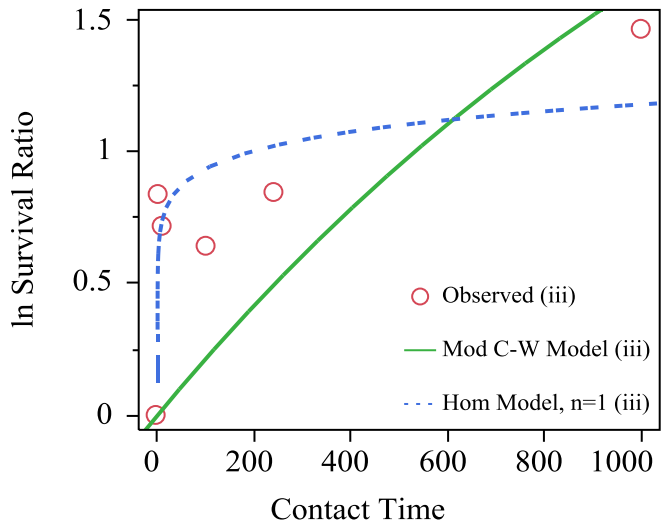

Figure 35: Model-predicted inactivation curves for *C. parvum* oocysts with 5 mg/L dose of free chlorine in buffered HDF water (Trial iii)

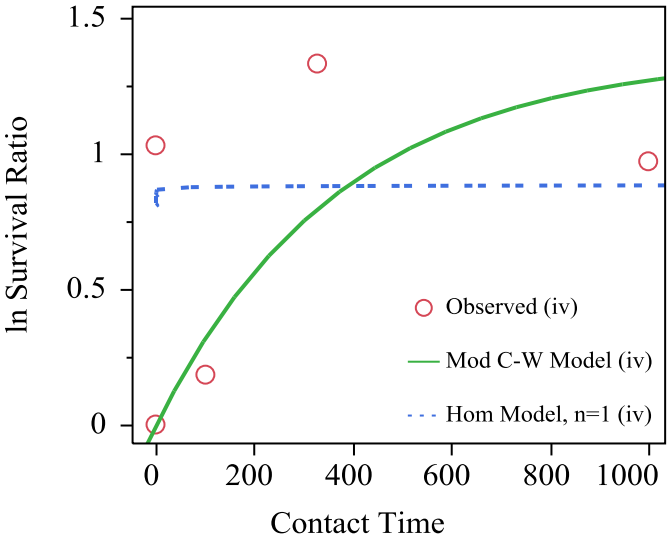

Figure 36: Model-predicted inactivation curves for *C. parvum* oocysts with 5 mg/L dose of free chlorine in buffered HDF water (Trial iv)

Disinfection by bromine shows an obvious departure from first-order kinetics. Therefore, the Hom model was tested first. The resulting parameters, mean squared error, and time to achieve 50% and 75% inactivation as predicted by the model are provided in Table 19.

Table 19: Hom model parameters, mean squared error (MSE), and time to achieve 50% and 75% *C. parvum* oocyst inactivation by a 5 mg/L dose of bromine in buffered HDF water

Trial	$k \pm$ SE	$m \pm$ SE	C_0 [mg/L]	MSE	$T_{50\%}$ [min]	$T_{75\%}$ [min]
i	0.158 ± 0.085	0.04 ± 0.11	4.1	0.21	6.8	--
ii	0.244 ± 0.125	0.00 ± 0.10	4.3	0.07	0.0	--
iii	0.282 ± 0.124	0.10 ± 0.09	3.6	0.43	0.0	22
iv	0.124 ± 0.023	0.16 ± 0.03	3.4	0.01	22	1,538

Trial	$k \pm$ SE	$m \pm$ SE	C_0 [mg/L]	MSE	$T_{50\%}$ [min]	$T_{75\%}$ [min]
iv	0.19 ± 0.07	0.05 ± 0.08	4.0	0.15	0.3	--

The parameter m was well below 1 for all trials, indicating a declining rate of inactivation (tailing-off kinetics). For trials ii and iii, the Hom model predicted an essentially instantaneous 50% decrease in oocyst infectivity, followed by a much slower inactivation rate. Bromine could not achieve 75% inactivation in Trials i and ii. In Trials iii and iv, the time to 75% inactivation were 22 and 1,538 minutes, respectively. The latter time lies outside the data of observations, so extrapolation may not provide a reliable estimate.

As seen in Figure 31, the bromine inactivation curves suggest the possibility of two sub-populations in the oocyst batch—one being less susceptible to bromine disinfection than the other. Therefore, the One Hit—Two Population (OHTP) model was applied to the bromine disinfection data. The OHTP formula was as follows:

$$\frac{N}{N_0} = f * e^{(-k_1 * t)} + (1 - f) * e^{(-k_2 * t)} \qquad (16)$$

where f is the fraction of the population subject to the rate constant k_1, while the quantity $(1-f)$ is the fraction of the population subject to the rate constant k_2. The resulting parameters, mean squared error, and time to achieve 50% and 75% inactivation as predicted by the model are provided in Table 20. In JMP, the parameter f was constrained to the interval from 0 to 1.

Table 20: One Hit—Two Population model parameters, mean squared error (MSE), and time to achieve 50% and 75% *C. parvum* oocyst inactivation by a 5 mg/L dose of bromine in buffered HDF water

Trial	$f \pm$ SE	$k_1 \pm$ SE	$k_2 \pm$ SE	MSE	$T_{50\%}$ [min]	$T_{75\%}$ [min]
i	0.40 ± 0.09	-0.0006 ± 0.0003	1.07 ± 0.82	0.026	1.7	--
ii	0.29 ± 0.06	-0.0002 ± 0.0003	1.62 ± 5.4E+18	0.005	0.8	--
iii	0.14 ± 0.07	-0.0004 ± 0.0007	0.22 ± 0.11	0.011	3.9	9.2
iv	0.45 ± 0.02	0.0006 ± 0.0001	1.62 ± 2.4E+18	0.000	1.5	1,038

	$f \pm$ SE	$k_1 \pm$ SE	$k_2 \pm$ SE	MSE	$T_{50\%}$ [min]	$T_{75\%}$ [min]
Bromine	0.28 ± 0.04	-.0004 ± 0.0002	0.32 ± 0.11	0.004	3.7	--

In Trials i-iii, the model predicted a sharp decrease in infectivity at the beginning of the experiment, followed by a much slower increase. In terms of parameters, this means that $k_1 < 0$ and $k_2 > 0$, and $|k_2| \gg |k_1|$, where k_1 represents the resistant oocyst population and k_2 represents the susceptible population. This is an interesting result, and possible reasons for this trend are discussed in Section 5.3.

The two models are compared in the following figures for each trial. In each figure, the OHTP model is plotted in the upper position, and the Hom model is plotted in the lower position. These models require different ordinate scales, so the left ordinate

axis plots $\frac{N_T}{N_0}$ and the right ordinate axis plots $\ln\left(\frac{N_T}{N_0}\right)$. The two models share the same abscissa, which plots contact time in minutes, as usual. The OHTP model is a superior model both by the MSE metric and by plausible theory. This is further discussed in Section 5.3.

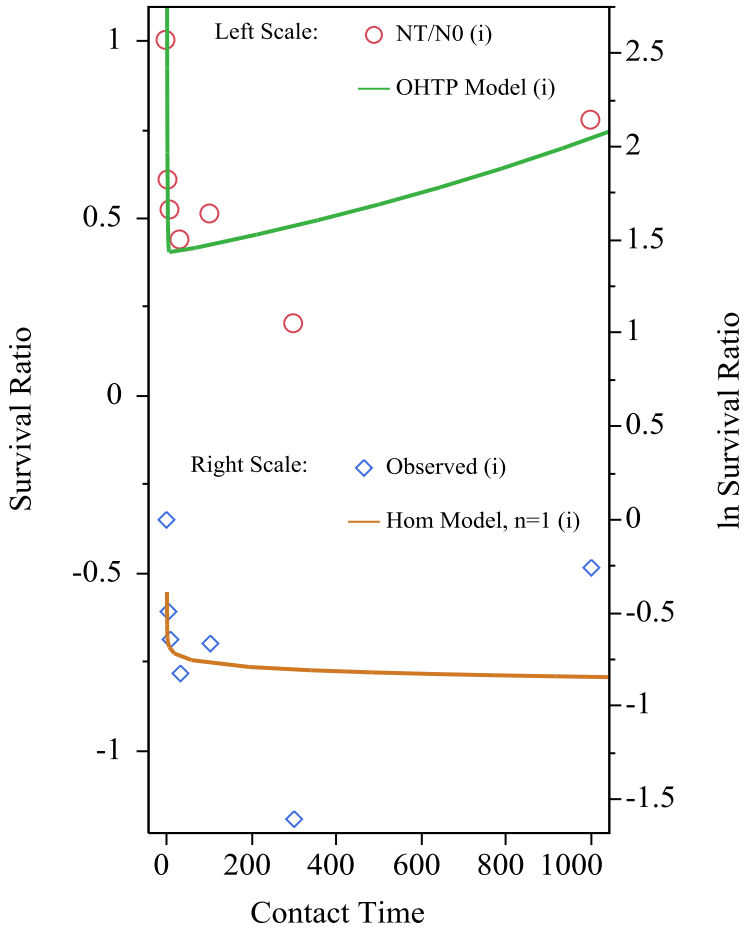

Figure 37: Model-predicted inactivation curves for *C. parvum* oocysts with 5 mg/L dose of free bromine in buffered HDF water (Trial i)

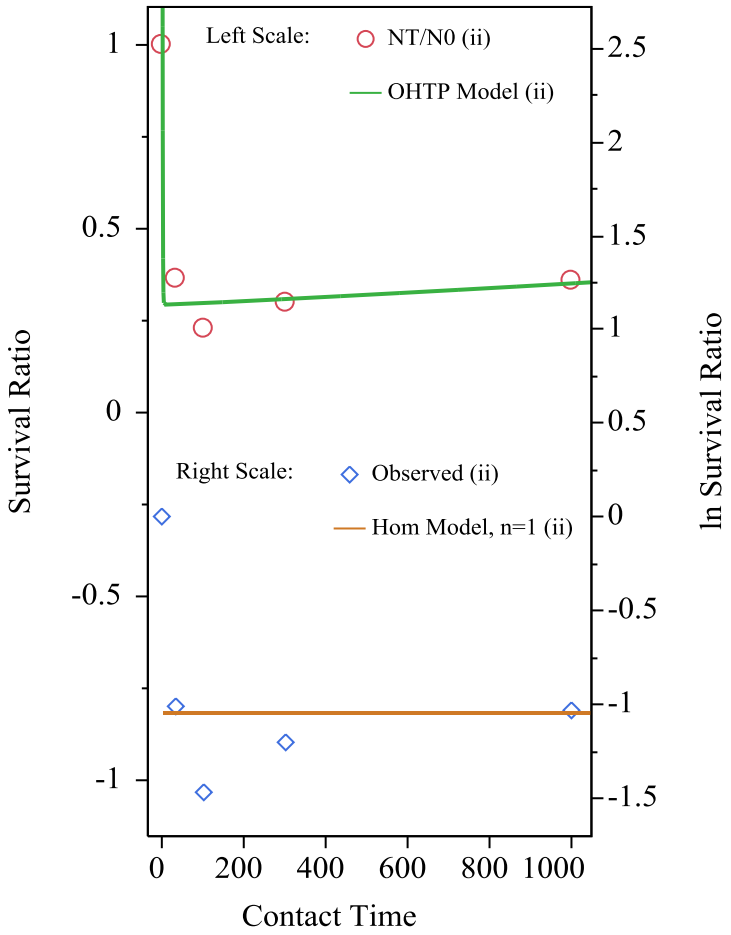

Figure 38: Model-predicted inactivation curves for *C. parvum* oocysts with 5 mg/L dose of free bromine in buffered HDF water (Trial ii)

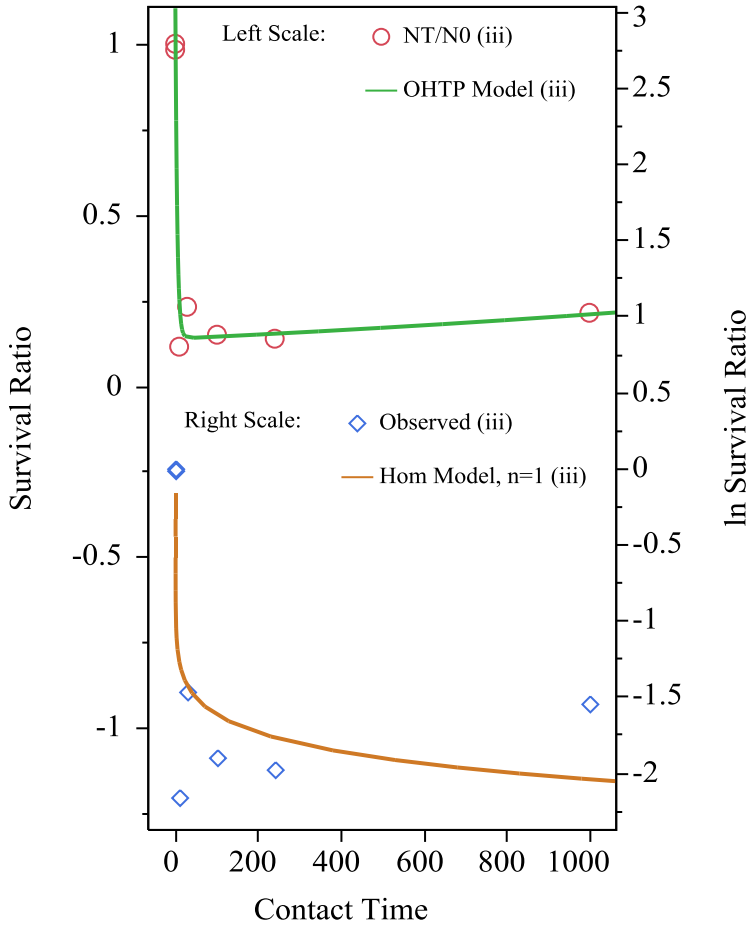

Figure 39: Model-predicted inactivation curves for *C. parvum* oocysts with 5 mg/L dose of free bromine in buffered HDF water (Trial iii)

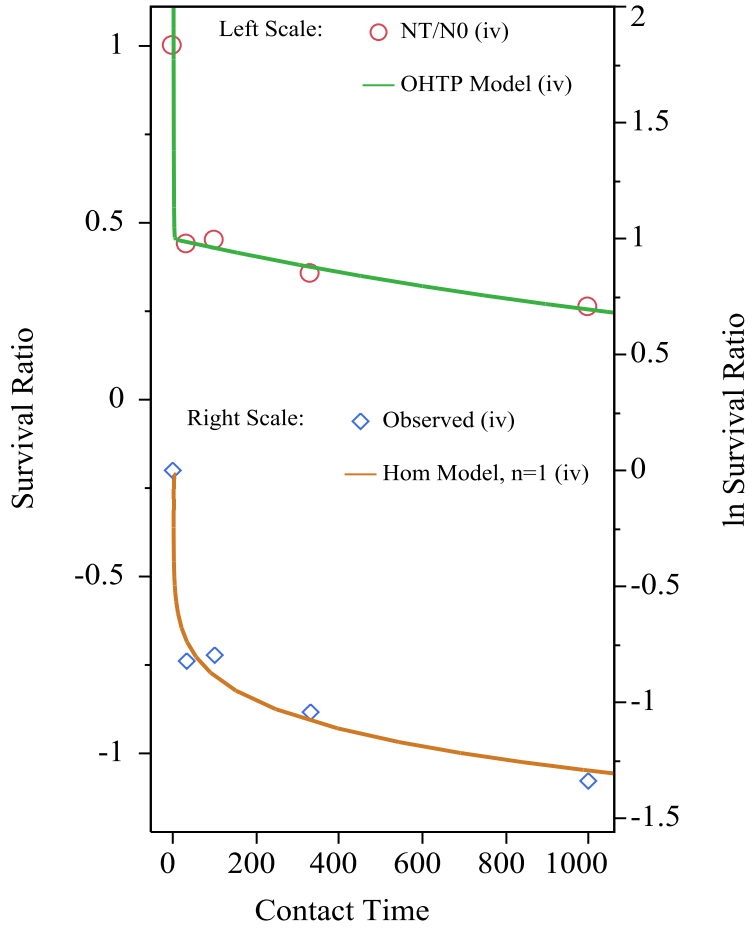

Figure 40: Model-predicted inactivation curves for *C. parvum* oocysts with 5 mg/L dose of free bromine in buffered HDF water (Trial iv)

5 DISCUSSION

The stated objectives of the research presented in this report were twofold: (1) to compare the efficacies of free chlorine and bromine in the disinfection of MS-2 coliphages, *B. atrophaeus* spores, and *C. parvum* oocysts; and (2) to investigate suitable models for the kinetics of inactivation of these microorganisms. These objectives were addressed in Section 4. The current section interprets the findings and their associated implications and provides recommendations for further research areas.

5.1 DISINFECTION OF MS-2 COLIPHAGES

Different classes of microorganisms respond to disinfection in different ways. Generally, vegetative bacteria are the most susceptible to disinfection, followed by viruses, and finally the bacterial spores and protozoan cysts (Sobsey, 1989). As expected, MS-2 (a virus) was the most susceptible of the three test organisms to halogen treatment. Its inactivation also happened to be the simplest to model of the three. MS-2 inactivation was rapid for both halogens and exhibited first-order inactivation kinetics; CT values to achieve 4 log10 reduction were $<$3.8 min*mg/L and 19 $\pm$ 2.5 min*mg/L for chlorine and bromine, respectively. At a dose of 5 mg/L, this translates to a required contact time of about 45 seconds and 3.8 minutes for chlorine and bromine, respectively.

The rate constant k, for MS-2 inactivation by chlorine was calculated to be 2.9 L/(mg*min), which is in close agreement with data in the literature. For instance, Haas et al. (1996) reported a rate constant of 3.4 L/(mg*min) in buffered demand-free water. The

pH of that experiment was 6.9, so the mole fraction of HOCl to OCl^- was much greater than in this research (at pH 7.5), and so a more efficacious disinfection would have been expected and was indeed shown in Haas et al. (1996).

The results of bromine disinfection also corroborated with similar research. Lindley (1966) tested the disinfection efficacy of free bromine on f2 phages (a different virus, but same class of organism) and found that a 4 mg/L dose of bromine achieved about 4.5 $\log_{10}$ reduction in 20 minutes in phosphate buffered water (0°C). The work presented in this report found that bromine required about 4 minutes of contact time to achieve a 4 $\log_{10}$ reduction in MS-2 (25°C). The elevated temperature in this experiment may explain the difference in rates, as it is well-known that an increase in temperature causes an increase in reaction rate constants.

5.2 DISINFECTION OF B. ATROPHAEUS *SPORES*

The inactivation of *B. atrophaeus* spores always exhibited an initial shouldering pattern. Shouldering is often associated with aggregated particles that subsequently get broken up during the reaction. The Hom model proved to be the best fit, with $m > 1$, which is indeed an indication that there may have been spore aggregation, resulting in this shouldering effect. However, the TWEEN® 80-amended experiment seems to refute this hypothesis because the shouldering effect was seen there too. It is plausible that spore aggregation still occurred in the TWEEN® 80-amended waters (albeit to a lesser degree), causing the initial shouldering. It is helpful to remember that water microorganisms tend to aggregate in the environment, and so the results of this work may actually resemble the real-world situation.

Concerning equimolar halogen disinfection experiments, it was somewhat unexpected to find that chlorine was more efficacious than bromine, as previous literature has suggested that free bromine was more virucidal than equimolar concentrations of free chlorine (Section 2.2.3). However, these comparisons are influenced by differences in other key variables because they are being made between different microorganisms, viruses and *Bacillus* spores and different lab groups that operated at different test conditions, including different pH values.

5.3 DISINFECTION OF C. PARVUM *OOCYSTS*

One puzzling aspect of the *C. parvum* oocyst infectivity assay was mentioned in Section 4.2.4, namely, the low excystation rates of the oocyst batches. In all four trials involving *C. parvum* oocysts, the assays of non-halogen-containing reaction vessel samples (i.e., where excystation should theoretically not be influenced) revealed excystation rates of no more than 0.1% (i.e., only 1 out of every 1,000 oocysts excysted and successfully infected the monolayer of HCT-8 cells). An excystation rate closer to 50% was expected, as this was typical for previous studies in the Sobsey lab (correspondence with Dr. Otto D. Simmons, III, NCSU). However, because the excystation rates of the control samples assayed in experimentation were *consistent* (albeit low), the reasons for low excystation rates were not explored further for this report.

The oocyst disinfection trials revealed complex inactivation kinetics. The increase in excystation of oocysts exposed to chlorine treatment was expected, as sodium hypochlorite is cited by several lab groups as a pre-treatment step that enhances oocyst excystation *in vitro* (Reduker & Speer, 1985) or infectivity *in vivo* (neonatal mice). The enhanced excystation is often attributed to the weakening of the oocyst outer wall,

facilitating the excystation of the internal infectious sporozoites. These results are different from those of a previous study where *C. parvum* oocysts experienced almost 90% inactivation when treated with 4.96 to 8.48 mg/L of free chlorine (Corona-Vasquez, et al. 2002). However, these previous studies used an in vitro excystation "viability" assay method that did not employ a direct assay of infectivity in either cell cultures or experimental animals. Therefore, a direct comparison of excystation results from those experiments to results from the current experiments based on oocyst infectivity is not possible.

The downward concave nature of the increase in infectivity of oocysts by chlorine treatment could not be well modeled by the models normally applied in inactivation kinetics studies, except for the Hom model. In practice though, it may not be necessary to model the oocyst infectivity increase with much precision, as the data suggest that there may be an upper limit to this increase. It may be sufficient just to recognize that chlorine at a 5 mg/L dose exhibits no disinfecting qualities toward *C. parvum* oocysts.

In contrast to chlorine, a 5 mg/L dose of bromine reduced the infectivity of oocysts. It may be that the oocyst wall is altered by bromine exposure beyond the point that is beneficial for future excystation in the presence of susceptible host cells. That is, successful excystation in the subsequent presence of host cells does not occur. Alternatively, brominated oocysts may excyst earlier than those treated with chlorine, resulting in early release of sporozoites into test waters where they then become inactivated before they ever get inoculated into cell cultures for infectivity assay. As an example, King et al. (2011) found that the infectivity of *C. parvum* sporozoites decreased dramatically if they did not encounter host cells in time to infect them. If sporozoites are

"leaking" out of damaged oocysts during bromine disinfection (i.e., prematurely), they will not have the opportunity to find a host cell to infect before they die or are otherwise destroyed during their susceptible life stage.

For disinfection by bromine, the One Hit—Two Population model was an attractive model, not just mathematically as seen in Section 4.2.4.1, but also mechanistically. The presence of two distinct populations of oocysts, distinguished by their susceptibility to halogens, is plausible. For instance, the fraction of oocysts that were quickly inactivated by bromine would correspond to the susceptible population, while the fraction of oocysts that slowly became more infectious would correspond to the more hardy population. This slow rise in infectivity could be explained by the same phenomenon seen in the slow rise in infectivity of the hardy population in chlorine treatment. In other words, the oocysts can be divided into two populations—one susceptible, and the other hardy. Chlorine induces excystation enhancement in the susceptible population perhaps by way of weakening the oocyst wall, while bromine reduces infectivity perhaps by excessive damage to the wall that causes premature leakage of sporozoites. Then, chlorine and bromine induce the same response in the hardy population, namely a slow rise in infectivity, as the halogens slowly weaken the oocyst wall, thereby facilitating successful excystation.

5.4 IMPLICATIONS FOR CENTRALIZED WATER TREATMENT

If, as hypothesized in Section 5.3, a susceptible oocyst sub-population exists, then the 2-$\log_{10}$ (99%) removal requirement of *Cryptosporidium* by filtration (Long Term 2 Enhanced Surface Water Treatment Rule – 40 CFR 141.700-141.722) may need to be reassessed for plants using free chlorine specifically, because when the requirement was

stipulated, it was not anticipated that oocysts become more infectious in chlorinated systems. However, results from the present cell culture infectivity study suggest that conventional treatment plants that chlorinate their water could actually be enhancing the infectivity of *C. parvum* oocysts that pass through their systems.

The findings of this research also have specific implications for municipalities that have bromide-impacted source waters, such as in coastal areas where saltwater intrusion is common and other areas with brackish groundwater. If these municipalities are utilizing free chlorine as their drinking water disinfectant, the chlorine will rapidly oxidize the bromides to free bromine (Section 2.2.2). This will alter the kinetics of microbial inactivation as free bromine is now an active disinfectant. Depending on the relative concentrations of bromide ions and free chlorine molecules, free bromine may either be working in concert with free chlorine or predominating as the operative disinfectant. The latter is likely according to Westerhoff et al. (2004), who found that "bromine reacts faster and substitutes more efficiently than chlorine." This is consistent with the finding that, even at low concentrations, the bromide ion has profound effects on speciation of byproducts in chlorine-disinfected water (Hua et al., 2006). Bromide is commonly present in source waters; Amy et al. (1994) report that bromide concentration in source waters in the U.S. average between 61 and 64 μg/L, while Kranser et al. (1996) report bromide concentrations in the range of 100-500 μg/L in source water samples from the California State Water Project, an area with historically high bromide concentrations.

Chlorination plant operators will have understandably modeled their disinfection process with chlorine inactivation kinetics, but they may need to review their process if

their influent water is bromide-impacted, especially since bromine was found to be less efficacious than chlorine in the disinfection of MS-2 coliphages and *Bacillus* spores.

5.5 RECOMMENDATIONS FOR FUTURE RESEARCH

Elucidating the mechanism of action for the reduction and enhancement of *C. parvum* excystation is the most important next step in this research. This can possibly be addressed by using vital dyes that are specific to living stages of *Cryptosporidium*. Then, phase contrast or differential interference contrast microscopy could be used to visually identify the physical nature of the oocysts at different exposure times. In theory, the snapshots of each time sample could be used to draw inferences about the mechanism of action.

The existence of a clear dose-response relationship is quite plausible, as this is the case for many organism-disinfectant combinations. Indeed, this dose-response relationship is the physical manifestation of Chick's law (where n=0). However, the current work cannot provide evidence of the disinfection efficacy of bromine and chlorine at doses other than the one tested. Therefore, it would also be good to test different doses of both bromine and chlorine, to learn if they retain their disinfecting power at lower doses. Testing higher doses would not be recommended, because 5 mg/L is the upper limit according to WHO guidelines. Lower doses are more common and therefore should be used in subsequent experiments.

As mentioned in Section 2.2.2, the literature has consistently shown that the hypohalous acid species is a stronger disinfectant than the hypohalite ion species. In other words, the disinfection efficacy of bromine and chlorine is affected by the pH of the water being treated. The pH of influent waters has a typical range of about 6 to 8. This

research only tested water at a pH of 7.5, the pK_a of chlorine, meaning chlorine existed as HOCl and OCl^- in equal molar parts. The literature suggests that testing at a pH of 6 (i.e., lower) will make disinfection more favorable for chlorine, and testing at a pH of 8 (i.e., higher) will make it less favorable, while bromine disinfection would remain relatively unaffected because HOBr predominates until pH 8.3, the pK_a of bromine. It would be especially interesting to see how the pH would affect the oocysts' susceptibility to treatment. Therefore, testing above and below pH 7.5 is recommended.

6 CONCLUSIONS

1. Chlorine and bromine were shown to adequately inactivate MS-2 coliphages and *Bacillus atrophaeus* spores in a model system under controlled experimental conditions at a dose of 5 mg/L. Both halogens were able to achieve greater than a 5-log_{10} reduction in culturable microorganisms within one minute of contact time.
2. At the same experimental conditions, chlorine and bromine were shown to be inadequate disinfectants against *Cryptosporidium parvum* oocysts at a dose of 5 mg/L. Neither was able to provide the EPA-regulated 2-log_{10} reduction in infectious oocysts, and in fact did not even provide 1-log_{10} reduction.
3. Chlorine was found to be a more efficacious disinfectant than bromine against both MS-2 coliphages and *B. atrophaeus* spores on an equal mass concentration basis, and was further found to be a more efficacious disinfectant than bromine against *B. atrophaeus* spores on an equimolar basis as well.

4. The Chick-Watson model was a sufficient model for the disinfection of MS-2 coliphages. MS-2 inactivation was rapid for both halogens and exhibited first-order inactivation kinetics; CT values to achieve 4 $\log_{10}$ reduction were $<$3.8 min*mg/L and 19 $\pm$ 2.5 min*mg/L for chlorine and bromine, respectively.
5. The disinfection of *B. atrophaeus* spores by chlorine and bromine exhibited shouldering characteristics and was well-modeled by the Hom model ($n = 1, m > 1$). At a 5 mg/L dose, chlorine and bromine achieved 4 $\log_{10}$ reduction in spores at around 32 and 440 minutes, respectively.
6. Chlorine treatment at 5 mg/L enhanced *C. parvum* oocyst infectivity according to the cell culture infectivity assay. A two-fold increase in infectivity occurred within 5 minutes, but the rate of "activation" quickly declined thereafter. This trend was best described by the Hom model ($n = 1, m < 1$).
7. Bromine treatment at 5 mg/L decreased oocyst infectivity. 50% reduction was achieved quickly within 4 minutes, followed by a very slow increase in infectivity. The largest observed reduction was 84%. The data were best modeled by the One Hit—Two Population theory, suggesting the presence of two oocyst populations with different levels of susceptibility to bromine.

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APPENDIX

HALOGEN RESIDUAL PLOTS

The following two figures track the halogen residual measurements over the duration of Trials ii and iii; the halogen residual plots for Trials i and iv have already been provided in the main text (Section 4.2.1). The open markers indicate single halogen readings from vessels containing no microorganisms, and the closed markers indicate those from microorganism-containing vessels. Error bars represent the manufacturer's 95% confidence interval for instrument readings ($\pm$ 0.2 mg/L). R^2 values are displayed as a goodness-of-fit indicator.

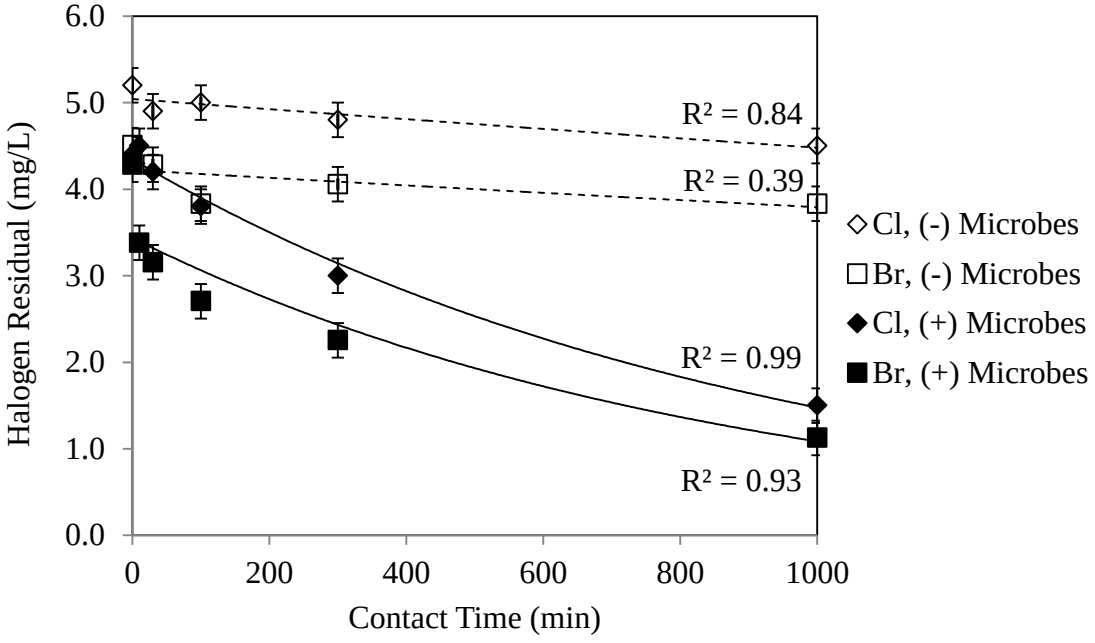

Figure 41: Halogen residual over time in HDF buffered water in reactors with and without test microbes (Trial ii)

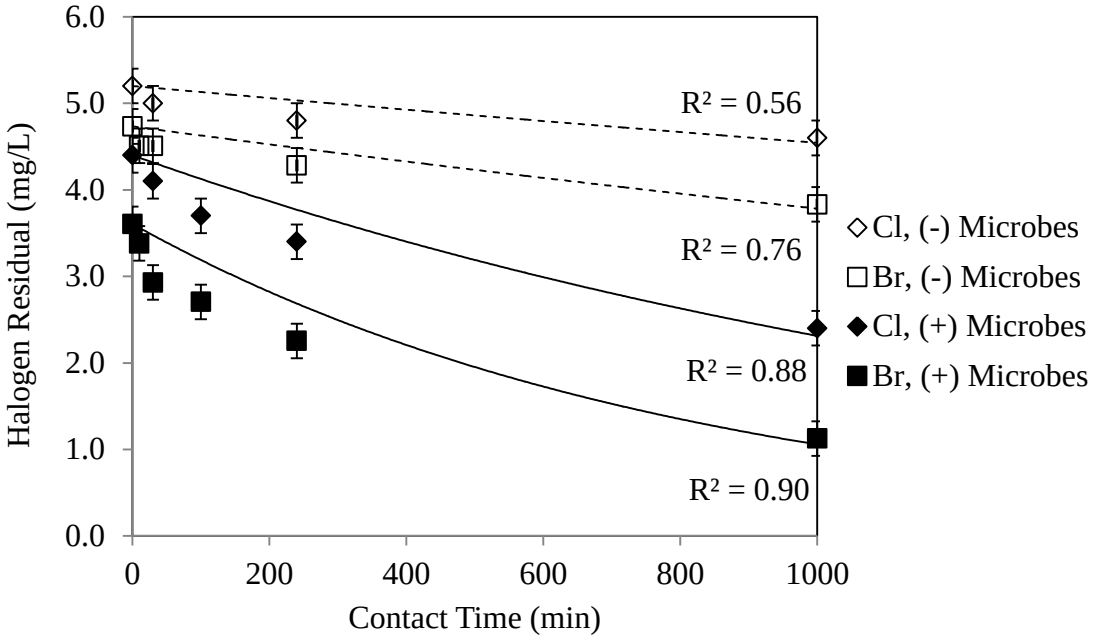

Figure 42: Halogen residual over time in buffered HDF water in reactors with and without tests microbes (Trial iii)

MICROBIAL SURVIVAL DATA TABLES

The following series of tables provides microbial survival counts of all trials. Any microbial concentration marked with an asterisk (*) represents the lower detection limit of the trial (i.e., the microorganism was not detectable). The $\log_{10}$ survival ratio computed from such entries therefore represents the minimum extent of inactivation.

Table 21: MS-2 survival in 5 mg/L halogen disinfection (Trial i)

Time (min)	Control	Cl, (+) MS-2		Br, (+) MS-2	
	PFU/mL	PFU/mL	$\log_{10}$ Survival R.	PFU/mL	$\log_{10}$ Survival R.
0	7.67E+04	1.27E+05		4.22E+05	
1	4.11E+04	* 5.56	< -4.36	* 5.56	< -4.88
10	8.56E+04	* 5.56	< -4.36	* 5.56	< -4.88
30	7.67E+04	* 5.56	< -4.36	* 5.56	< -4.88
100	9.61E+04	* 5.56	< -4.36	* 5.56	< -4.88
300	5.17E+04	* 5.56	< -4.36	* 5.56	< -4.88
1000	1.16E+05	* 5.56	< -4.36	* 5.56	< -4.88

Table 22: MS-2 survival in 5 mg/L halogen disinfection (Trial ii)

Time (min)	Control	Cl, (+) MS-2		Br, (+) MS-2	
	PFU/mL	PFU/mL	Log_{10} Survival R	PFU/mL	Log_{10} Survival R
0	1.44E+04	1.39+E+04		2.17E+04	
1	1.00E+04	* 5.56	< -3.40	* 5.56	< -3.59
10	3.17E+04	* 5.56	< -3.40	* 5.56	< -3.59
30	1.22E+04	* 5.56	< -3.40	* 5.56	< -3.59
100	1.67E+04	* 5.56	< -3.40	* 5.56	< -3.59
300	3.22E+04	* 5.56	< -3.40	* 5.56	< -3.59
1000	3.28E+04	* 5.56	< -3.40	* 5.56	< -3.59

Table 23: MS-2 survival in 5 mg/L halogen disinfection (Trial iii)

Time (min)	Control	Cl, (+) MS-2		Br, (+) MS-2	
	PFU/mL	PFU/mL	Log_{10} Survival R	PFU/mL	Log_{10} Survival R
0	1.04E+06	1.16E+06		1.57E+06	
1		* 5.56	< -5.32	* 5.56	<-5.45
10	9.28E+05	* 5.56	< -5.32	* 5.56	< -5.45
30	1.14E+06	* 5.56	< -5.32	* 5.56	< -5.45
100	1.77E+06	* 5.56	< -5.32	* 5.56	< -5.45
240	1.01E+06	* 5.56	< -5.32	* 5.56	< -5.45
1000	1.35E+06	* 5.56	< -5.32	* 5.56	< -5.45

Table 24: MS-2 survival in 5 mg/L halogen disinfection (Trial iv)

Time (min)	Control	Cl, (+) MS-2		Br, (+) MS-2	
	PFU/mL	PFU/mL	Log_{10} Survival R	PFU/mL	Log_{10} Survival R
0	6.11E+05	7.17E+05		6.89E+05	
1		* 5.56	< -5.11	* 5.56	< -5.09
10		* 5.56	< -5.11	* 5.56	< -5.09
30		* 5.56	< -5.11	* 5.56	< -5.09
100	4.17E+05	* 5.56	< -5.11	* 5.56	< -5.09
300	4.72E+05	* 5.56	< -5.11	* 5.56	< -5.09
1000	7.78E+05	* 5.56	< -5.11	* 5.56	< -5.09

Table 25: MS-2 survival in 5 mg/L halogen disinfection (Trial v)

Time (min)	Control	Cl, (+) MS-2		Br, (+) MS-2	
	PFU/mL	PFU/mL	Log_{10} Survival R	PFU/mL	Log_{10} Survival R
0	1.83E+06	1.44E+06		2.32E+06	
1		* 5.56	< -5.40	2.08E+05	-1.05
3		* 5.56	< -5.40	7.28E+04	-1.50
6		* 5.56	< -5.40	* 5.56	< -5.62
10		* 5.56	< -5.40	* 5.56	< -5.62
30	2.12E+06	* 5.56	< -5.40	* 5.56	< -5.62

Table 26: *B. atrophaeus* spore survival in 5 mg/L halogen disinfection (Trial i)

Time (min)	Control	Cl, (+) Spores		Br, (+) Spores	
	CFU/mL	CFU/mL	Log_{10} Survival R	CFU/mL	Log_{10} Survival R
0	1.44E+04	2.11E+04		1.83E+04	
1	7.78E+03	1.06E+04	-0.30	5.89E+03	-0.49
10	3.89E+03	3.67E+03	-0.76	2.56E+03	-0.86
30	4.44E+03	* 5.56	< -3.58	4.11E+03	-0.65
100	6.11E+03	* 5.56	< -3.58	2.28E+03	-0.91
300	1.00E+04	* 5.56	< -3.58	* 5.56	< -3.52
1000	1.44E+04	* 5.56	< -3.58	* 5.56	< -3.52

Table 27: *B. atrophaeus* spore survival in 5 mg/L halogen disinfection (Trial ii)

Time (min)	Control	Cl, (+) Spores		Br, (+) Spores	
	CFU/mL	CFU/mL	Log_{10} Survival R	CFU/mL	Log_{10} Survival R
0	[a]2.36E+05	[b]2.39E+05		[c]2.59E+05	
1	2.36E+05	2.39E+05	0.00	2.59E+05	0.00
10	1.89E+05	9.55E+04	-0.40	2.55E+05	-0.01
30	1.13E+05	3.03E+01	-3.90	2.79E+05	0.03
100	1.73E+05	* 5.56	< -4.63	1.73E+05	-0.17
300	2.40E+05	* 5.56	< -4.63	2.34E+03	-2.04
1000	1.87E+05	* 5.56	< -4.63	* 5.56	< -4.67

[a] Observed concentration (1.34E+05) has been replaced by the next concentration in the time series.
[b] Observed concentration (1.51E+05) has been replaced by the next concentration in the time series.
[c] Observed concentration (1.39E+05) has been replaced by the next concentration in the time series.

Table 28: *B. atrophaeus* spore survival in 5 mg/L halogen disinfection (Trial iii)

Time (min)	Control	Cl, (+) Spores		Br, (+) Spores	
	CFU/mL	CFU/mL	Log_{10} Survival R	CFU/mL	Log_{10} Survival R
0	1.94E+04	[a]3.11E+04		[b]3.83E+04	
1		3.11E+04	0.09	3.83E+04	0.06
10	1.83E+04	1.05E+04	-0.38	4.06E+04	0.09
30	1.89E+04	* 5.56	< -3.65	2.44E+04	-0.13
100	1.11E+04	* 5.56	< -3.65	2.06E+04	-0.21
240	1.11E+04	* 5.56	< -3.65	7.06E+03	-0.67
515				16.7	-3.30
1000	1.56E+04	* 5.56	< -3.65	* 5.56	< -3.78

Table 29: *B. atrophaeus* spore survival in 5 mg/L halogen disinfection (Trial iv)

Time (min)	Control	Cl, (+) Spores		Br, (+) Spores	
	CFU/mL	CFU/mL	Log_{10} Survival R	CFU/mL	Log_{10} Survival R
0	9.11E+05	6.78E+05		8.78E+05	
1		4.50E+05	-0.18	7.06E+05	-0.09
10		4.33E+05	-0.19	6.44E+05	-0.13
30		5.78E+02	-3.07	2.39E+05	-0.57
100	7.56E+05	* 5.56	< -5.09	3.11E+05	-0.45
300	4.22E+05	* 5.56	< -5.09	2.93E+05	-0.48
515				3.25E+05	-0.43
1000	3.67E+05	* 5.56	< -5.09		

Table 30: *B. atrophaeus* spore survival in 5 mg/L bromine or 2.2 mg/L chlorine disinfection (Trial vi)

Time (min)	Control	Cl, (+) Spores		Br, (+) Spores	
	CFU/mL	CFU/mL	Log_{10} Survival R	CFU/mL	Log_{10} Survival R
0	1.17E+06	1.23E+06		1.59E+06	
1		8.44E+05	-0.16	1.22E+06	-0.11
10		1.03E+06	-0.08	1.66E+06	0.02
30		1.37E+05	-0.95	1.52E+06	-0.02
100		55.6	-4.34	9.44E+05	-0.23
300	1.12E+06	* 5.56	< -5.34	50.0	-4.50
970	1.94E+06	* 5.56	< -5.34	* 5.56	< -5.46

[a] Observed concentration (2.50E+04) has been replaced by the next concentration in the time series.
[b] Observed concentration (3.33E+04) has been replaced by the next concentration in the time series.

Table 31: *C. parvum* oocyst infectivity in 5 mg/L halogen disinfection (Trial i)

Time (min)	Control		Cl, (+) Oocysts		Br, (+) Oocysts	
	IU/mL stock	Log_{10} Survival Ratio	IU/mL stock	Log_{10} Survival Ratio	IU/mL stock	Log_{10} Survival Ratio
0	659		497		642	
1	837	0.10	1098	0.34	437	-0.17
10	625	-0.02	827	0.22	374	-0.23
30	879	0.12	1205	0.38	327	-0.29
100	788	0.08	827	0.22	361	-0.25
300	827	0.10	1599	0.51	156	-0.62
1000	625	-0.02	1262	0.40	504	-0.10

Table 32: *C. parvum* oocyst infectivity in 5 mg/L halogen disinfection (Trial ii)

Time (min)	Control		Cl, (+) Oocysts		Br, (+) Oocysts	
	IU/mL stock	Log_{10} Survival Ratio	IU/mL stock	Log_{10} Survival Ratio	IU/mL stock	Log_{10} Survival Ratio
0	768		1073		1124	
1			2042	0.28		
10						
30					482	-0.37
100			1744	0.21	314	-0.55
300			2474	0.36	388	-0.46
1000	1000	0.11	2474	0.36	445	-0.40

Table 33: *C. parvum* oocyst infectivity in 5 mg/L halogen disinfection (Trial iii)

Time (min)	Control		Cl, (+) Oocysts		Br, (+) Oocysts	
	IU/mL stock	Log_{10} Survival Ratio	IU/mL stock	Log_{10} Survival Ratio	IU/mL stock	Log_{10} Survival Ratio
0	688		575		868	
1			1001	0.24	878	0.00
10			1098	0.28	174	-0.70
30					229	-0.58
100	641	-0.03	1012	0.25	151	-0.76
240			1036	0.26	139	-0.80
1000	679	-0.01	2071	0.56	208	-0.62

Table 34: *C. parvum* oocyst infectivity in 5 mg/L halogen disinfection (Trial iv)

Time (min)	Control		Cl, (+) Oocysts		Br, (+) Oocysts	
	IU/mL stock	Log_{10} Survival Ratio	IU/mL stock	Log_{10} Survival Ratio	IU/mL stock	Log_{10} Survival Ratio
0	574		516		464	
1						
10	893	0.19				
30					205	-0.36
100	731	0.11	621	0.08	209	-0.35
330	893	0.19	1946	0.58	164	-0.45
1000	874	0.18	1366	0.42	122	-0.58